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JOURNAL OF DAIRY SCIENCE

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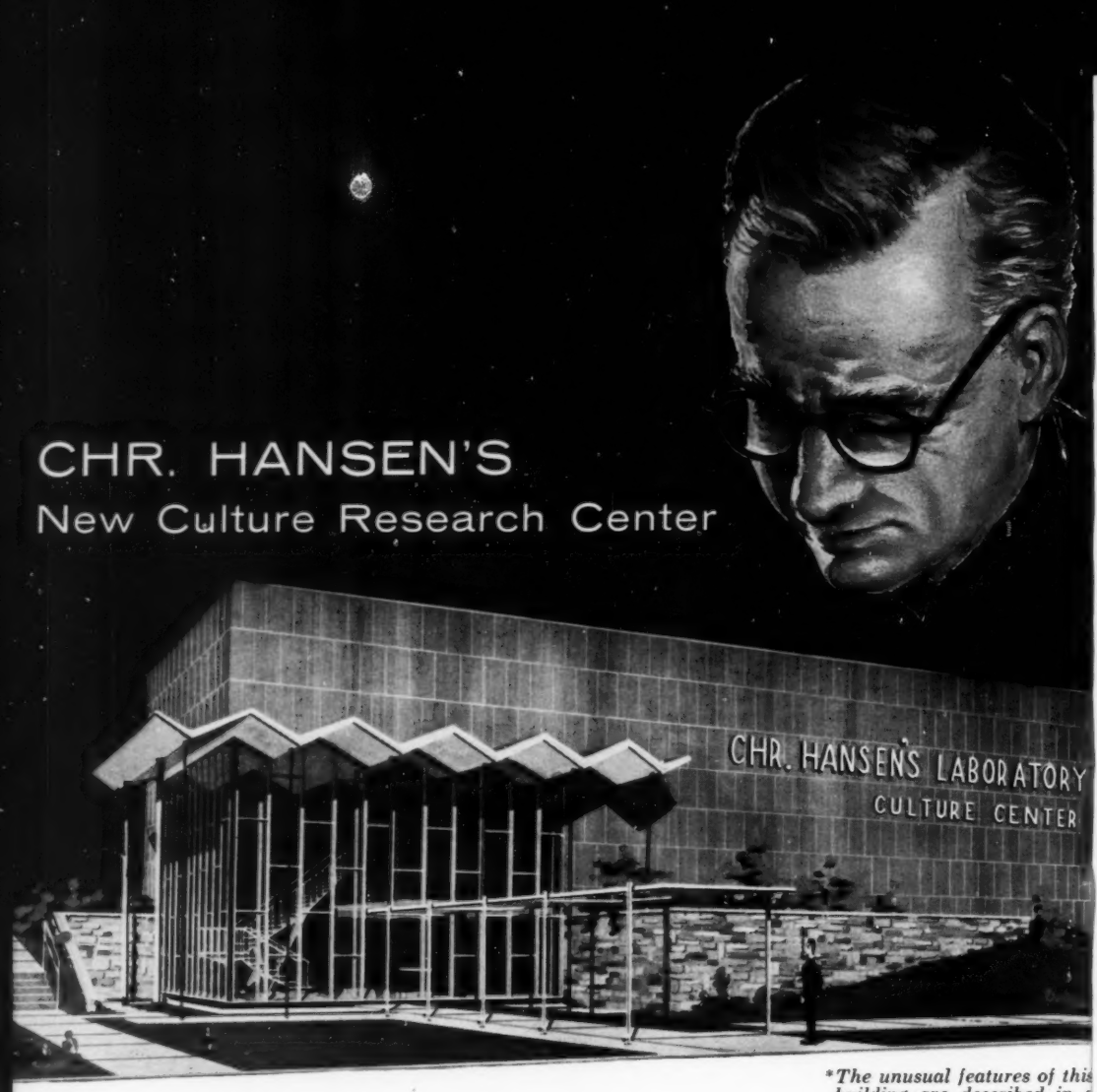

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PEOPLE AND EVENTS

John G. Archibald Retires

On June 30, 1961, J. G. ARCHIBALD, Research Professor in Animal Nutrition at the University of Massachusetts, completed 40 years of service at that Institution. His research accomplishments were in grass silage, trace minerals, and forage quality. He was honored on January 26 at the Annual Massachusetts Dairy Farmers' Seminar with a citation read and presented by Dr. D. J. Hankinson, Head of the Department of Dairy and Animal Science. Professor Archibald had previously presented a paper entitled, Forty Years in Retrospect.[1]

Professor J. G. Archibald with Mrs. Archibald receiving the citation from Dr. D. J. Hankinson.

On June 8, a farewell party was given Professor and Mrs. Archibald by members of the Dairy and Animal Science Department at the Yankee Peddlar Inn, Holyoke, Massachusetts. At this time, an original water color painting by the Amherst artist, Stephen Hamilton, was presented to them.

The Archibalds will spend their summers at their cottage in West Chesterfield, New Hampshire, and winters in Florida. Their Florida mail address is c/o John M. Archibald, 491 West 32nd Place, Hialeah, Florida.

All of their friends joined in wishing the Archibalds a happy and healthful retirement.

CITATION

JOHN GEDDIE ARCHIBALD

PROFILE OF A RESEARCHER

We have just enjoyed hearing your account of Forty Years in Retrospect. Those of us involved in planning the 11th annual Dairy Farmers' Seminar could think of no better occasion to publicly acknowledge your own part in these 40 years of progress. We know of your plans to retire in June and we wish to salute your many years of faithful, fruitful service.

Some of the people in the audience do not know your entire background. You see, while 40 years have been spent here, my research points out the fact that 40% of your years have been accumulated elsewhere. Before some aspiring mathematician computes your age from these data, I will point out that you were born June 11, 1894, in Cavendish, Prince Edward Island, the son of a Presbyterian minister. Not knowing anything about Prince Edward Island as a suitable birthplace qualifying one for research work, I can project the theory that your birthright, because of environment or inheritance, imparted the high degree of integrity, which has been evidenced in your work.

You earned the Bachelor of Science degree from Ontario Agricultural College in 1916, an institution highly regarded in the United States. You were Assistant Chemist at the Nova Scotia Agricultural College from 1916 to 1920 and Research Chemist for the International Nickel Company from 1920 to 1921. You joined the staff of the Massachusetts Agricultural College in 1921 and earned the Master of Science degree in 1924.

To inject a nostalgic note, these were times when names such as Kenyon L. Butterfield (President); Joseph B. Lindsey (Experiment Station); John C. Graham (Poultry); Christian I. Gunness (Agricultural Engineering); Fred C. Sears (Pomology); Walter W. Chenoweth (Horticultural Manufactures); Charles E. Marshall (Bacteriology); Henry S. Green (Librarian); Sidney B. Haskell (Director of Experiment Station); and Fred C. Kenney (Treasurer) represented campus leaders.

But, John, time has a way of speeding on. Your accomplishments have been many. Your field of interest has been animal nutrition, as your presentation has emphasized. We look upon your research with grass silage, trace minerals, and forage quality as outstanding, and you have not by any means confined your attention to these fields.

Normally, most men contemplating retirement start to decelerate well ahead of the stopping point so that all in view are aware of the action. Not so with you! Your attention to your feeding trials has kept your schedule full and, in addition, three scientific articles have been published in 1960, with two more manuscripts in progress.

[1] This paper will be published in Our Industry Today section of the JOURNAL.

To prove that your horizons have not been limited by the walls of your laboratory, we point to your home—an example of Christian living in the very best sense. You married Cora Blenkhorn, a Massachusetts girl, in 1916. You have been active in the Congregational Church of Amherst. You and Mrs. Archibald have raised four children who, with grandchildren, are now strategically located from California to Florida—this, no doubt, to facilitate a retirement with travel.

But most of all, John, we appreciate your friendly nature, your helpfulness, and your understanding of human nature. You have been a loyal member of your Department, a devoted public servant, and friend to all of us. The dairy farmers of Massachusetts so testify as you stand before them.

/s/ Rodman C. Nowers
Head Farmer, Medfield State Hospital

/s/ D. J. Hankinson
Head, Department of Dairy and Animal Science

/s/ F. P. Jeffrey
Acting Dean, College of Agriculture

Dr. F. N. Andrews Appointed Department Head at Purdue

Dr. F. N. Andrews, internationally known animal physiologist and head of Purdue University's dairy department, will become head of Purdue's new Department of Animal Sciences, January 1, 1962. This will combine the staffs and the programs formerly administered in the Departments of Animal Science, Poultry Science, and Dairy. Dr. J. H. Martin, head of the Poultry Science department, will be assistant head of the newly created department and will also coordinate genetic investigations in the enlarged department. Professor Claude Harper, who for the last 16 years has headed the Department of Animal Science, has received leave from January 1, 1962, to June 30, 1962—date of his official retirement. He will leave late in January for Indonesia, for an assignment under the International Co-operation Administration.

F. N. Andrews

Dr. Andrews is an internationally recognized authority on fertility and sterility of cattle, sheep, swine, and horses; physiology of growth of all species and types of farm and laboratory animals; and the interrelations of physiology, genetics, nutrition, and pathology of animals.

The new department head earned the B.S. and M.S. degrees from the University of Massachusetts in his home state and the Ph.D. degree from the University of Missouri. He served on the staffs of both universities before coming to Purdue. In 1949, he was advanced to Professor of Animal Science and from 1949 to 1953 was assistant to the Dean of the Purdue graduate school. Dr. Andrews is a member of Phi Kappa Phi, Gamma Sigma Delta, and Sigma Xi.

Dale K. Weber and Jewell M. Jensen honored at Michigan Dairy Manufacturers' Conference

At the Annual Dairy Manufacturers' Conference at Michigan State in November notable recognition was accorded two of the active members of the industry.

D. K. Weber

J. M. Jensen

D. K. Weber, General Manager of Heatherwood Farms Dairy, Lansing, was the recipient of the Michigan Dairy Manufacturers' Award for 1961. J. M. Jensen, Associate Professor of the new department of Food Science, Michigan State University, received recognition on his twenty-fifth year of service to the dairy industry of the state.

Mr. Weber graduated in 1933 from Iowa State University. For a time he worked in the University creamery. Later he was with Robert's Dairy Company at Sioux City. He came to Heatherwood Farms Dairy, Lansing, in 1941 as plant superintendent, advancing in 1957 to general manager. During all his service in Michigan he has actively participated in the programs of the local and state organizations of dairy processors. He has served as president of the Michigan Dairy Products Association and on its numerous committees. He has appeared on many dairy technology programs in Michigan and other states. Currently, Mr. Weber is a member of the Advisory Committee guiding the newly estab-

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lished Department of Food Science at Michigan State. He has also been active in the civic affairs of his city, having served as president of the Lansing Sales Executive Club and in other organizations.

In the citation to Mr. Weber, presented by P. S. LUCAS, Professor Emeritus, a significant sentence reads, "Your capacity for sincere friendliness, your modesty, your public spirit, your unselfish devotion to the needs of the industry, your vision, your unusual ability as a leader, your approachability, thoughtfulness, and joy in the advancement of others mark you as a fine example of a gentleman and responsible leader."

Mr. Weber's enlarged photograph is now displayed in the Industry Conference Room in the dairy building at the university. He thus joins other leaders who have been previous recipients of the award.

J. M. Jensen, who has served the dairy industry so effectively in Michigan, along with Mrs. Jensen, was quite abashed in the recognition they received. Dairy companies, associations, and individuals in Michigan subscribed to a fund for them to make a trip to Denmark next September. Professor Jensen is scheduled to present a paper, Some Flavor Problems with Pipeline and Bulk-Handled Milk at the XVIth International Dairy Congress to be held in Copenhagen, September 3-7, 1962.

Mrs. Jensen was presented a dozen red roses. Both received various gifts to make travel most pleasing. The travel certificate they were given indicated that the fund carried the cash value of $2,538.51. Subsequently, the sum has been increased by additional contributions.

The citation presented to Professor Jensen by D. B. FALCONER of Sealtest Foods, Detroit, reads in part, "You have brought to the classroom and laboratory not only your native Danish dedication to the high quality of dairy products but a range of experience and practical approach to dairy problems as well."

Professor Jensen was born in Denmark. His family, including the older brother, Chris, who is at North Dakota Agricultural College, came to North Dakota when the boys were quite young. They settled on a farm in that state. Professor Jensen has never returned to visit the country of his birth.

When he finished high school in North Dakota, the budding young dairyman chose to study Dairy Industry at Iowa State University, where his uncle, the late Doctor M. Mortensen, was head of the Dairy Industry Department.

Graduating at Iowa State in 1928, Mr. Jensen came to Michigan State for his Master's study. After completing that work he was employed for five years at the Soo Creamery at Sault Ste. Marie. Then he came to

Michigan State University as Extension Specialist in Dairy Manufactures, in which work he rendered notable service to dairy plants and organizations. This is reflected in their eagerness to participate in the recent recognition for him.

In 1941 he was changed from extension to resident work. He has made notable contributions in research; for his teaching he is acclaimed by many former students. His successes in coaching the Dairy Products judging teams have been notable.

J. C. Knott Dairy Center Dedicated at Washington State University

The new seven-structure dairy research and teaching unit at Washington State University was dedicated as the J. C. Knott Dairy Center in recognition of 38 years of service to the university.

Dr. C. C. FRENCH, WSU president, told the 250 people attending the ceremony that the Board of Regents changed a longtime policy to name buildings after a person still living.

Dr. L. L. MADSEN, director of Agricultural Sciences at WSU, said that during Dr. Knott's 38 years of service to the university, he made many contributions to the dairy industry and to agriculture generally in the state, the Northwest, and in the United States and Latin America.

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He traced Dr. Knott's record from his student days at WSU and his first job with the university as dairy herdsman, through his rise up the academic ladder to full professor of dairy science and then director of the Agricultural Extension Service, and the Institute of Agricultural Sciences.

He also lauded Dr. Knott's service on many regional and national dairy science and U. S. Department of Agriculture advisory committees, and the international demand for Dr. Knott's services as a judge and advisor on dairy problems. He has judged dairy cattle in Canada, Ecuador, and Colombia, and has been honored by the Washington Dairy Hall of Fame.

Veterinary Associations Honor C. F. Huffman

C. F. HUFFMAN, who retired July 1, 1961, from his position as Professor in the Dairy Department at Michigan State University, has been further honored by two veterinary associations.

C. F. Huffman

At the annual meeting of the Michigan Veterinary Medical Association on Mackinac Island, Doctor Huffman was made an Honorary Member.

Then, at the recent annual meeting of the American Veterinary Medical Association, he was made an Honorary Member of that association. The plaque presented to him on that occasion reads "for his distinguished service to veterinary science and to the advancement of its application."

These two significant honors for Doctor Huffman are highly cherished among the many accorded him.

Russell E. Horwood Dies

R. E. HORWOOD, 59, retired, died in September at his home in Lake City, Michigan.

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Professor Horwood had retired in April after 34 years of work in the Dairy Department and the Cooperative Extension Service of Michigan State University. A serious heart condition suffered in October, 1960, led to the necessity that he retire. (J. Dairy Sci., August, 1961.)

R. E. Horwood

Professor Horwood was born and reared on a farm in Livingston County, an area widely known for excellent dairy herds and dairymen. After completing the work for his Master's degree, he served 10 years as Dairy Extension Specialist in the Upper Peninsula, with headquarters at the Chatham Experiment Station, where he was able to conduct many research projects of value. In 1938 he was recalled from Chatham to serve on the Dairy Staff at East Lansing. Then, in 1949 he was reassigned back to the Upper Peninsula as superintendent of the station at Chatham and district extension supervisor for the entire U.P.

Twice in Professor Horwood's career he was delegated to serve on foreign assignments. During 1947 he served in Japan for the United States Army to help organize an agricultural extension service there. In 1951 he was assigned by Michigan State as the first director of the MSU mission to establish the University of the Ryukyus on Okinawa. He served for two years on that assignment.

Dr. T. H. Bartilson Dies

T. H. BARTILSON, assistant director of the Animal Husbandry Research Division of the U. S. Department of Agriculture at Beltsville, Md., died October 9 in the Prince Georges General Hospital after a long illness. He was 62.

T. H. Bartilson

Born in Braddock, Pa., he received his B.S. degree from Pennsylvania State University in 1921. In 1923 and 1924 he taught poultry husbandry at the University of Maryland, from which he received the degrees of M.S. in 1941 and Ph.D. in 1950.

Before joining USDA in 1929, Dr. Bartilson was agricultural extension agent in Cecil County, Maryland.

He was a member of Phi Kappa Phi, Alpha Zeta, and Pi Sigma Alpha, all honorary agricultural societies, the American Society for

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SUMMARY

TERRAMYCIN IN MILK PRODUCTION
AVERAGE OF 17 TRIALS (186 DAYS)

	Control	Treated
Number of cows	1193	1177
Av. days on experiment [1]	186	186
Av. daily milk prodn./cow, lb.	30.98	31.85
Increase in daily production, lb.	—	0.87
Increase, %	—	3
Extra milk per cow, lb.	—	161.8
Value of extra milk, $[2]	—	8.09
Cost of Terramycin per cow, $[3]	—	1.26
Extra return per cow, $	—	6.83

[1]Range 119-280 days [2]Milk valued at $5 per cwt.
[3]Terramycin 13.95 gm./cow; value $0.09/gm.

There was no effect of Terramycin feeding upon the bacterial content of milk, the antibiotic did not appear in the assayed milk, and cheese cultures were not inhibited by this milk.

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the Advancement of Science, and the American Society of Animal Production. He was also a 32nd degree Mason and a Shriner.

Dr. Bartilson leaves his wife, the former Ruth Brigham of the home address, Hillmeade Road, Bowie, Md., and two brothers, E. W. Bartilson of Beallsville, Ohio, and George H. of East McKeesport, Pa.

He is also survived by four sisters, Mary I. Sarver of New Wilmington, Pa.; Margaret E. Murdoch of Woodland, Mich.; Edith Carol Chapman of Wilkinsburg, Pa., and Helen Jane Phillips of Edgewood, Pa.

Services were held on October 12, with burial in Arlington National Cemetery.

Michigan News

The following short courses and conferences have been organized at Michigan State University:

Quality control and analysis of milk and milk products, with DR. J. R. BRUNNER in charge, January 22 to February 2, 1962; Milk and related products, DR. G. M. TROUT in charge, February 5 to 16, 1962; Dairy Engineering, PROFESSOR A. RIPPEN in charge, February 19 to March 2, 1962.

The Ninth Annual National Dairy Engineering Conference, DR. C. W. HALL in charge, will be held February 27 to 28, 1962. The conference will be devoted to automation components for the dairy industry.

Ohio News

Dairy Technology Homecoming Brunch

Over 150 alumni of the Ohio State University's Department of Dairy Technology and their guests attended the Thirteenth Annual Dairy Technology Homecoming Brunch, held at Columbus, on November 4.

In an atmosphere of football music and displays depicting the Department's current and past activities, the alumni enjoyed a prebrunch social period, where old acquaintances with classmates and faculty were renewed. After the brunch, served by members of the student Dairy Tech Club, D. G. WALLAR, '42, Mojonnier Bros. Company, Columbus, served as Master of Ceremonies for the formal program. The high light of the program was the presentation of the 1961 Dairy Technology Award of Merit to F. S. HARTER, Chairman of the Board of Reiter and Harter, Inc., of Akron, by DR. R. M. KOTTMAN, Dean of the College of Agriculture and Home Economics.

F. S. Harter

Mr. Harter was cited by Dean Kottman for his outstanding contributions to the Ohio Dairy Industry and for his dedicated service to the people of his community and of his state. His early association with the Dairy Industry in a family business led to his founding a company which, after a merger with Reiter's Dairy of Akron, has grown into one of Ohio's larger and more progressive independent dairy companies. He is Vice-President and President-Elect of the Ohio Dairy Products Association, and will direct the affairs of that important organization as President in 1962. He is also a member of the Board of Directors of the United Dairy Cooperative Association of Mason City, Iowa. Mr. Harter's public service has included four terms in the Ohio House of Representatives and three in the Ohio Senate, where he served as Chairman of the Finance Committee. As a legislator, he demonstrated an objective awareness of the problems of the Dairy Industry and of Agriculture as a whole, and by applying with keen insight the same constructive, realistic leadership qualities which brought him success in business, he made major contributions to the solution of these pressing problems. Last year Mr. Harter was elected to his first term as Summit County Commissioner and has recently been appointed by the Governor as the public representative on the Capital Planning Commission. He has been a member of Kiwanis since 1919, and is a member of the Christian Church in Akron.

Dairy Technology Societies Meet

It was Student Night when the Northeastern Ohio, the Maumee Valley, and the Cincinnati Dairy Technology Societies met on November 8, 9, and 16, respectively. Each year this event constitutes the first formal activity in the cooperative program between the Ohio Dairy Technology Societies and the Department of Dairy Technology, The Ohio State University, designed to attract students to a Dairy Industry Career. High school students, attending the meetings as guests of Society members, were shown a slide sequence depicting the opportunities represented by a career in the Dairy Industry and the facilities and educational program of the Department. A tour of a local dairy plant also was an important part of each program. At the Northeastern meeting, the students toured the Chestnut Ridge Dairy in Akron; students at the Maumee Valley meeting visited Driggs Dairy in Toledo; and at Cincinnati, the processing facilities of the Kroger Company were visited.

The Central Ohio Dairy Technology Society met on November 8, in Columbus. Featured speaker was B. M. Saffian, Associate Director, Chamberlain Laboratories at Stow, Ohio, Mr. Saffian discussed the topic, Plastics and Their Application to the Dairy and Food Industries.

Virginia News

W. S. GRIFFITH is on leave from Virginia Polytechnic Institute to pursue graduate work leading toward his doctorate at the University of Chicago. Added to the staff to take Griffith's place is J. A. LINEWEAVER, a 1961 graduate of the Department of Dairy Science. He will be working on the Dairy Science Extension Program. Lineweaver is listed in Who's Who Among Students in American Colleges and Universities, is a member of A.D.S.A., Alpha Zeta, and Phi Sigma. He received the Borden Scholarship and the Ralston Purina undergraduate award. At the 1961 A.D.S.A. annual meeting he was elected president of the National Student Affiliate Organization.

The fifth annual Dairy Science Career Day was held on November 3 on the V.P.I. campus. Twenty-five carefully selected high school students and ten sponsors attended the meeting. High light of the program was a talk by WILLIAM KISER, a 1947 graduate of the department. He discussed career opportunities for Dairy Science graduates.

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West Virginia News

Southern Dairy Products Contest Held at the University

Eight teams competed in the Southern Dairy Products contest on October 20 at the University of West Virginia: Clemson, Georgia, Louisiana, Mississippi, Oklahoma, Texas Tech, V.P.I. and the host, West Virginia.

High teams in All Products were Texas Tech, Mississippi, and Oklahoma. The high individuals were: GARY COWELL, West Virginia; WINSTON WAGGONER, Texas Tech; and DAN WOODRUFF, Mississippi.

Mississippi was first in Milk, with Gary Cowell (West Virginia) high individual. Frst honors in Butter went to Texas Tech, with Winslow Waggoner of that institution high man; Cheese was won by Georgia, with RALPH HILL of V.P.I. best individual, and Louisiana State took the cup in Ice Cream, with JERRY MOORE of Clemson the winner of the gold charm.

The W.V.U. Dairy Science Club (DR. ALLEN RAKES, Adviser) sponsored an Open House for the contestants which included a tour of the new facilities. On the afternoon following the contest, DR. RALPH MITCHELL led a tour of a glass factory, the new Medical Center and the Dairy Farm. PROFESSOR S. J. WEESE coordinated the contest and DR. ROBERT THOMAS the Awards Banquet.

New Agricultural Sciences Buildings at the University

The Dairy Science Department recently moved into new and greatly expanded facilities in the Agricultural Sciences Building on the Evansdale Campus. Modern offices for staff, graduate students, conference, reading and work rooms, as well as teaching and research laboratories, are included in the new layout. The dairy plant on the ground floor is equipped with the most up-to-date processing machines and C.I.P. lines.

With the new equipment, the Department is prepared to accept more graduate students at the Master's level, and plans are rapidly developing for a program leading to a Ph.D. in the animal field.

The new three-building Agricultural Center will be dedicated on Monday, January 29, 1962. The ceremony will be the opening event of a three-day, Living Resources Forum for Agriculture, Forestry, and Home Eocnomics. DR. I. D. PORTERFIELD, Chairman of the Department of Dairy Science, is chairman of a committee responsible for developing the forum, which will feature some of the nation's agricultural leaders as speakers.

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Industry News

Beatrice Foods Expands in Burma

Beatrice Foods Co. signed contracts on November 20 to complete negotiations for the construction of a $2,500,000 milk plant in Rangoon, Burma, in a joint venture with the Burmese government and two other groups.

Beatrice Foods will supervise building of the plant, installation of equipment to be designed and built here, and will provide the engineering and technical advice for the company, which will be known as Beatrice Foods (Burma) Ltd.

The Burmese Defense Services Institute (a Burmese government corporation) will provide 70% of the capital. Beatrice Foods, Frazer & Neave, Ltd., a Singapore publicly owned beverage company in Malaya, and K. C. Leong of Kuala Lumpur, Malaya, and Aw Cheng Chye of Singapore will provide a total of 30%.

Promotions

H. R. ALEXANDER has been promoted to manager of Model Farms Dairy, a division of Beatrice Foods Co., in Louisville, Kentucky, and J. C. MATULENAS has been named to succeed him as manager of Beatrice Foods dairy plant operations in Waukegan, Illinois.

H. R. Alexander

J. C. Matulenas

Alexander started with the company's milk plant in Kankakee, Ill., in 1936 as a salesman. Six years later, he was named manager of Beatrice Foods' Meadow Gold plant in Waukegan, Ill.

Matulenas began his career with the firm in 1935 as a route driver-salesman for the Waukegan plant. He was promoted to a number of sales and supervisory positions and was appointed sales manager for the Waukegan Dairy operations in 1950.

Borden's Discontinue Operations at Dixon, Illinois

The Borden Foods Company discontinued the evaporated and condensed milk operations at its Dixon, Ill., plant on November 15 because of the decreasing demand for evaporated milk. Borden's will continue to receive Grade A milk at this plant from its regular suppliers.

The number of evaporated milk plants in the United States has dropped 41 per cent in the past 10 years, from a total of 122 in 1950 to 72 in 1960. Milk used in production of evaporated milk in the 10-year period has dropped 25 per cent, from nearly 6 billion pounds in 1950 to 4½ billion pounds in 1960. During this same period, per-person consumption of evaporated milk in this country has dropped 38%, from 18.1 lb in 1950 to 11.3 lb last year. Exports of the product have been decreased by more than 33% in the same period.

Ralph Meier Appointed Sales Manager at Dairyland

Dairyland Food Laboratories, Inc., Waukesha, Wisconsin, has announced the appointment of R. J. MEIER as Sales Manager of the company's new Veterinary Division. Mr. Meier will be responsible for sales and market development of PGE, Dairyland's new enzyme preparation for the treatment and prevention of calf scours. He graduated from the University of Wisconsin with a degree in Agriculture. He then entered the army and was commissioned an officer in the paratroops. Upon leaving the service, Mr. Meier went to work for Nutrena Mills as a sales representative. Most recently he was the sales representative for Pitman-Moore Laboratories in Eastern Minnesota and Western Wisconsin.

R. J. Meier

K. W. Ward Named Foremost Vice-President

K. W. WARD, general manager of the Industrial Division of Foremost Dairies, Inc., has been elected a vice-president of Foremost. He was president of Western Condensing at Appleton, Wisconsin, when it became a part of Foremost Dairies, Inc.

Dr. Marth Promoted at National Dairy Products

E. H. MARTH has been promoted to Senior Research Bacteriologist in the Fundamental Laboratory of the National Dairy research center in Glenview, Illinois.

Marth joined the company in 1957 as a bacteriologist. He is a graduate of the University of Wisconsin, where he majored in Bacteriology, earning his Doctorate Degree in 1954. He is a member of the American Society for

Microbiology, American Dairy Science Association, International Association of Milk and Food Sanitarians, and Institute of Food Technologists.

USDA

Dr. R. D. Plowman Assists Government of Spain

Under the U. S. Aid Program, DR. R. D. PLOWMAN spent two weeks in Spain during December. He helped plan a 200-cow dairy farm with milk processing facilities. The purpose of this project is to increase the milk supply, demonstrate improved dairy practices, and improve the quality of all milk supplies. It is hoped this aid program will encourage imports of cattle and feed grains from the United States.

Theses Completed

M.S. Degree:

NORMAN W. DURRANT—The effect of increasing the serum protein content of cottage curd on yield and quality. Virginia Polytechnic Institute.

WESLEY J. FENTON—Heat resistance of psychrophilic microorganisms. West Virginia University.

JAMES M. KEITH—The effect of physical form on the nutritive value of hay when fed to lactating cows. Virginia Polytechnic Institute.

Dairy Technology Societies

Central Illinois—Dr. Bruce Larson, Department of Dairy Science, University of Illinois, was the featured speaker at the December 6 meeting, held at the University of Illinois YMCA, Champaign. His subject: Impressions and Scenes from Russia.

Detroit—Speaker at the October meeting at Cregar's Pickwick House on Grand River was Dr. O. W. Kaufmann, of the Department of Microbiology and Public Health of Michigan State University.

Nebraska—Program for the November 15 meeting consisted of a talk by George H. Weaver, Liberty Glass Company, on the subject, Glass Containers—Past, Present, Future.

Philadelphia—Featured at the November 9 meeting was Samuel C. Tease, Vacuum Process Division, F. J. Stokes Corporation, Philadelphia. His talk dealt with Freeze Drying—Its Application to the Food Industry.

Tri-State—Christmas Party was held December 13 at the Fort Steuben Hotel Ballroom, Steubenville, Ohio. Dinner and dancing were enjoyed.

Western Michigan—October meeting was held at Finger's Cafe, Grand Rapids. Guest speaker was Charles Rosell, in charge of Development and Application Engineering, Chicago Stainless Equipment Corporation, on the topic What You Cannot Do with a Deodorizer.

ANIMAL PRODUCTION

Journal of The British Society of Animal Production

Contents of Volume 4, Part 1, February 1962.

COCKBURN, ELIZABETH O. John Hammond—the man.
HAMMOND, SIR JOHN. Selected Bibliography 1912-1960.
BOAZ, T. G., & ELSLEY, F. W. H. The growth and carcass quality of bacon pigs reared to different weights at 56 days old.
BUCK, S. F., HARRINGTON, G., & JOHNSON, R. F. Prediction of lean percentage of pigs of bacon weight from carcass measurements.
CALLOW, E. H. The relationship between the weight of a tissue in a single joint and the total weight of the tissue in a side of beef.
DICKERSON, G. E. Implications of genetic-environmental interaction in animal breeding.
DICKINSON, A. G., HANCOCK, J. L., HOVELL, G. J. R., TAYLOR, ST. C. S., & WIENER, G. The size of lambs at birth—a study involving egg transfer.
GREENWOOD, A. W. An experiment with a controlled environment for the domestic fowl.
HANCOCK, J. L., & HOVELL, G. J. R. Insemination before and after the onset of heat in sows.
HARRIS, L. E., & PHILLIPSON, A. T. The measurement of the flow of food to the duodenum of sheep.
JOUBERT, D. M. The maternal effects on size at birth and weaning in Landrace-native pig crosses.
SCHINCKEL, P. G. Variation in wool growth and of mitotic activity in follicle bulbs induced by nutritional changes.
SMITH, C., KING, J. W. B., & GILBERT, N. Genetic parameters of British Large White bacon pigs.
TAYLOR, ST. C. S. Identical twins and developmental stability.
TURNER, H. N., HAYMAN, R. H., TRIFFITT, L. K., & PRUNSTER, R. W. Response to selection for multiple births in the Australian Merino.

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—Russell Roberts
Trainee Sealtest Foods
Southern Division
Atlanta, Georgia

CONTENTS

SECTION I states the objectives and requirements of the program.

SECTION II contains training schedules for all phases of plant operation for all types of plants. Many questions are asked for the trainee to answer before passing from one phase of training to the next, and progress reports and rating forms are provided.

SECTION III covers management development. Many study projects are outlined which are not only essential to management development but may result in savings to the plant that will more than pay the cost of training.

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RESEARCH PAPERS

FACTORS OF VALVE DESIGN ASSOCIATED WITH HOMOGENIZATION OF MILK[1]

J. J. BETSCHER,[2] W. J. HARPER, AND D. A. SEIBERLING[3]
Department of Dairy Technology, The Ohio State University, Columbus

SUMMARY

Specially designed experimental valves with an extended-orifice construction were used to provide additional information concerning the mechanism of homogenization. Homogenization efficiency, determined by a centrifugal method, and the pressure drop across the valve, were utilized to evaluate valve performance.

The design of the experimental valve permitted alterations in construction making it possible to study, independently, factors related to homogenization. The factors investigated included: (a) the time required for a particle to pass through the valve clearance, (b) the velocity through the valve clearance, (c) the entrance and exit conditions of the valve, and (d) the effect of two or more valves in series.

At a given velocity, variations in valve orifice length from 0.10 to 1.6 in. had no effect on either pressure drop or homogenization efficiency. As the velocity through the valve of a fixed length was increased, the pressure drop increased and an improvement in homogenization efficiency was obtained. The use of a valve with a beveled entrance resulted in decreases in both pressure drop and homogenization efficiency, as compared to a valve with a sharp entrance. The same valve with the bevel at the exit had no effect on pressure drop.

When baffle plates were inserted following the valve exit at distances varying from 0.01 to 2.44 in., homogenization efficiency was improved with no change in pressure drop. The improvement was related inversely to the distance between the baffle plate and the valve exit.

The literature is in agreement that homogenization is physically achieved by greatly reducing the size of the fat globules. However, there are many theories as to the mechanism of this phenomenon, some of which are: explosion, impact, acceleration, and deceleration (11); shearing, viscous shear at the boundary vs. turbulent shear in the core (10); cavitation (3, 4), and wire drawing (15). At this time, no theory to explain homogenization has been universally accepted.

Previous work concerning the mechanism of homogenization has involved the use of standard or modified commercial homogenizer valves. This has resulted, in most cases, in the simultaneous study of a number of conditions. In addition, accurate control of variables such as valve clearance, velocity, and time of passage through the valve has not been possible.

The major objectives of this investigation were (a) to gain additional evidence regarding the mechanism of homogenization and (b) to determine the effect of valve design on homogenization efficiency.

Received for publication March 16, 1960.

[1] Technical Paper: 3:60. Department of Dairy Technology, The Ohio State University. Supported in part by the Ohio Dairy Products Research Fund.

[2] Present address: Pet Milk Co., Greenville, Ill.

[3] Present address: Klenzade Products Inc., Beloit, Wis.

EXPERIMENTAL PROCEDURE

An experimental homogenizer valve was designed which permitted extensive variations in the duration of the homogenization process, the flow velocity, the valve entrance and exit condition, and the number of stages employed. The valve consisted of a stainless steel plug drilled and reamed to provide an orifice diameter of 0.085 in. and spacers constructed of short sections of stainless steel tubing. The valve and valve assembly are shown in Figure 1. These parts were assembled and maintained in the position shown with bakelite gaskets and a locking handle. The experimental valve body was designed to replace the conventional first-stage valve on a 300 g.p.h. Cherry-Burrell Superhomo head. The

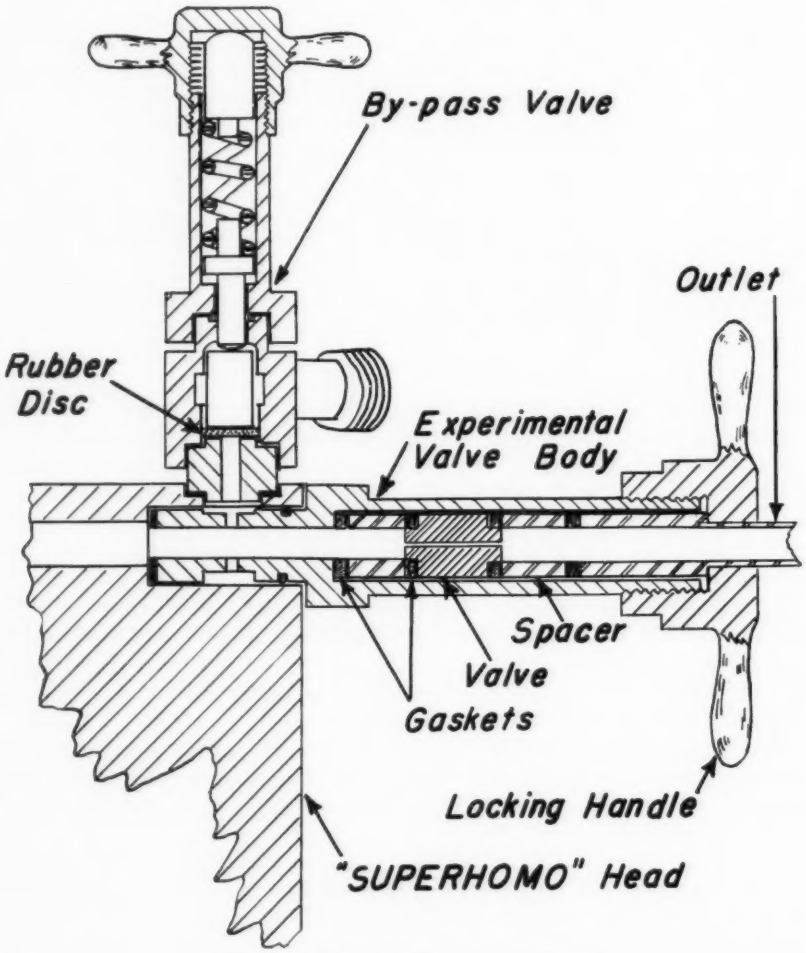

FIG. 1. Experimental valve and assembly.

second-stage valve of the Superhomo was modified to serve as a bypass valve by replacing the perforated valve caps with a flat rubber disc (approximately $\frac{3}{16}$-in. thick) between the valve plug and seat to divert the flow and to enable the homogenizer pump to be started at zero pressure.

Processing and sampling. Fresh mixed-herd milk was used in all experiments. The milk was standardized to approximately 4% fat, pasteurized at 150° F. for 30 min., and homogenized at 150° F. Samples were collected approximately 1 min. after the pressure became constant and cooled immediately to 40° F. Homogenization was completed within 45 min. after pasteurization. Preliminary trials showed that holding milk at 150° F. up to 60 min. had no effect on homogenization efficiency.

Flow-rates of 150, 300, and 450 gal/hr were obtained by connecting, in parallel, a Cherry-Burrell Viscolizer pump with a rated capacity of 150 gal/hr and a Cherry-Burrell Superhomo pump with a rated capacity of 300 gal/hr. This was accomplished by using a common suction line and connecting the discharge side of the two heads with No. 60 hydraulic hose.

The efficiency of homogenization was determined by a centrifugal method developed by Maxcy (5), as modified by Seiberling (8, 9). The method is based on induced fat rising by centrifugation, after which the fat content of 9 g. of the top 10 ml. is determined by a modified Babcock test. The homogenization efficiency index (HEI) is calculated as follows:

$$\text{HEI} = \frac{2\text{Fep} - \text{Fa}}{\text{Fa}} \times 100$$

Fep = Fat test of 9 g. of enriched portion (upper 10 ml.)

Fa = Average fat test of original samples

In calculating HEI the fat test of the enriched portion is multiplied by two to correct for the use of a 9-g. sample in an 18-g. Babcock test bottle.

This method yields a high HEI on poorly homogenized samples and a low value for well-homogenized products.

EXPERIMENTAL RESULTS

Effect of velocity, length of extended orifice, and passage time. Homogenization efficiency as related to velocity and passage time was studied by varying the flow rates from 150 to 450 gal/hr and the length of the orifice from 0.1 to 1.6 in.

At flow rates of 150, 300, and 450 gal/hr the corresponding velocities were calculated to be 142.1, 284.2, and 426.3 ft. per second, respectively. These velocities provided a range which encompassed the normal velocity through the valve clearance of 328 ft/sec, reported by Wittig (16). The velocities through the valves at the experimental flow rates were calculated by the following formula:

$$V = \frac{Q}{A}$$

Where: V = velocity (ft/sec)
Q = volume rate of flow (cu. ft/sec)
A = area (sq. ft.)

Using the calculated velocities, the time of passage was calculated as follows:

$$\theta = \frac{L}{V}$$

Where: θ = time (sec.)
L = length (ft.)

At a velocity of 284.2 ft/sec the mean orifice length (0.4 in.) provided a time of passage of approximately 1/10,000 sec., a time considered normal for passage through a valve clearance (16) (Table 1). At each flow rate there was a 16-fold increase in passage time produced by lengthening the orifice from 0.1 to 1.6 in. The extreme variations in time ranged from 20×10^{-5} sec. (approximately 1/50,000 sec.) to 93.9×10^{-5} sec. (approximately 1/1,000/sec).

At a given velocity, the HEI and pressure drop are independent of the time of passage or valve length (Table 1). Only minor variations occurred in pressure drops and HEI with valves of different lengths. No trends were apparent and the differences were within the experimental error of the methods used. Results shown in the last line of Table 1 are averages of pressure drops and HEI from 12 individual trials, disregarding the valve length for the three velocities studied. As the velocity increased from 142.1 ft/sec to 426.3 ft/sec, the average pressure drop increased from 444 lb/sq in. to 2,645 lb/sq in. and the HEI decreased from 686.9 to 78.4. These results indicate that both pressure drop and the HEI are dependent on velocity.

Effect of valve entrance conditions. A valve, 0.8-in. long, was beveled (60°) at one end to determine the effect of altered entrance or exit conditions. The maximum diameter of the bevel was 0.375 in. A standard valve with a sharp entrance, and having the same length of unmodified diameter as the beveled valve, was used as a control. Preliminary observations indicated that when the bevel was placed at the exit no change in pressure drop occurred, whereas a marked reduction in pressure drop was observed when the bevel was placed at

TABLE 1

Effect of passage time and length of extended orifices and velocity on pressure drop and efficiency of homogenization [a]

Length of orifice (in.)	Velocity (ft/sec)								
	142.1			284.2			426.3		
	Passage time sec. 10^{-5}	P [b]	EHI [c]	Passage time sec. 10^{-5}	P	EHI	Passage time sec. 10^{-5}	P	EHI
0.1	5.9	413	662.2	2.9	1,445	105.1	2.0	2,425	91.2
0.2	11.7	.....	.....	5.9	1,438	103.9	3.9	.....	.....
0.3	17.6	425	720.4	8.8	1,443	120.9	5.9	2,744	81.9
0.6	35.2	463	644.5	17.6	1,500	122.2	11.7	2,875	61.7
0.8	46.9	450	701.9	23.5	1,510	128.4	15.6	2,733	60.7
1.6	93.9	450	705.6	46.9	1,444	128.8	31.3	2,581	96.7
Average [d]		440	686.9		1,463	118.2		2,672	78.4

[a] Average of three trials.
[b] Pressure drop in lb/sq in.
[c] EHI—Efficiency of homogenization index; inversely related to efficiency.
[d] Average of 18 trials disregarding orifice length.

the valve entrance. Based on these observations, in further trials, the modified valve was used only with the beveled end as the entrance.

As indicated in Table 2, the use of a beveled entrance valve resulted in lower pressure drops and decreased homogenization efficiency compared to those obtained with a valve with a sharp entrance. However, at flow rates of 150 gal/hr and 300 gal/hr the decreased homogenization efficiency was less than would be expected for the pressure drop. For example, at 300 gal/hr the pressure drop was 900 lb/sq in. and the expected HEI should be approximately 400, based on results with the sharp-entrance valve. However, the average of the actual HEI was only 167. At the 450 gal/hr flow rate, the HEI was proportional to that expected on the basis of the pressure drop across the valve.

TABLE 2

Differences in pressure drop and homogenization resulting from the use of sharp and beveled valve entrances

		0.8 in. sharp		0.8 in. beveled	
Flow-rate	Lot	P [a]	EHI [b]	P [a]	EHI
(gal/hr)					
150	E-1	450	701.5	200	750.8
150	E-2	450	702.6	200	760.8
300	E-1	1,500	166.6	900	182.2
300	E-2	1,500	121.2	900	204.9
300	A	1,500	126.7	900	147.5
450	E-1	2,800	58.8	1,750	126.9
450	E-2	2,800	61.7	1,725	151.0

[a] Pressure drop in lb/sq in.
[b] EHI—Efficiency of homogenization index; inversely related to efficiency.

Effect of distance between baffle plates and valve exit. Baffle plates (as shown in Figure 2C) provided a means of studying the effect of variations in the conditions immediately following the valve exit upon the efficiency of homogenization. Baffle plates were located at distances varying from 0.01 to 2.44 in. from the exit of the 0.6-in.-long valve.

As revealed by representative results in Table 2, within the range investigated, the efficiency of homogenization was improved by decreasing the distance between the valve exit and the baffle plate. As the space was decreased from 2.44 to 0.01 in., the HEI decreased from 82.1 to 44.6. An HEI of 118.0 was obtained when the valve was operated without a baffle plate. The distance between the valve exit and the baffle had no measurable effect upon the pressure drop.

Effect of multi-stage valves. Multi-stage homogenization was obtained by placing two valves (0.2 and 0.3 in. long) in series and using a flow rate of 300 gal/hr. Preliminary results showed that the valves each caused a pressure drop of from 1,400 to 1,550 lb/sq in. when used alone. When the two valves were used in series, the total pressure drop across both valves theoretically should have been in the range of 2,800 to 3,000 lb/sq in. (12). However, as seen in Table 4, this theoretical pressure drop was not achieved. It was ap-

proached only when the space between the valves was 2.44 in., or when a baffle was used between the valves. Furthermore, results in Table 4 indicate that within the experimental error of the method, in two-stage as in single-stage homogenization, the HEI is related to pressure drop across the valve.

The effect of valves placed in series on pressure drop may be related to the flow pattern of the fluid. Hypothetical drawings illustrating possible flow patterns as affected by the distance between the valves are presented in Figures 2a and b. As suggested in Figure 2a, when valves are separated by a small distance most of the stream passes directly from the first to the second valve with only minor expansion and contraction. When the distance between the

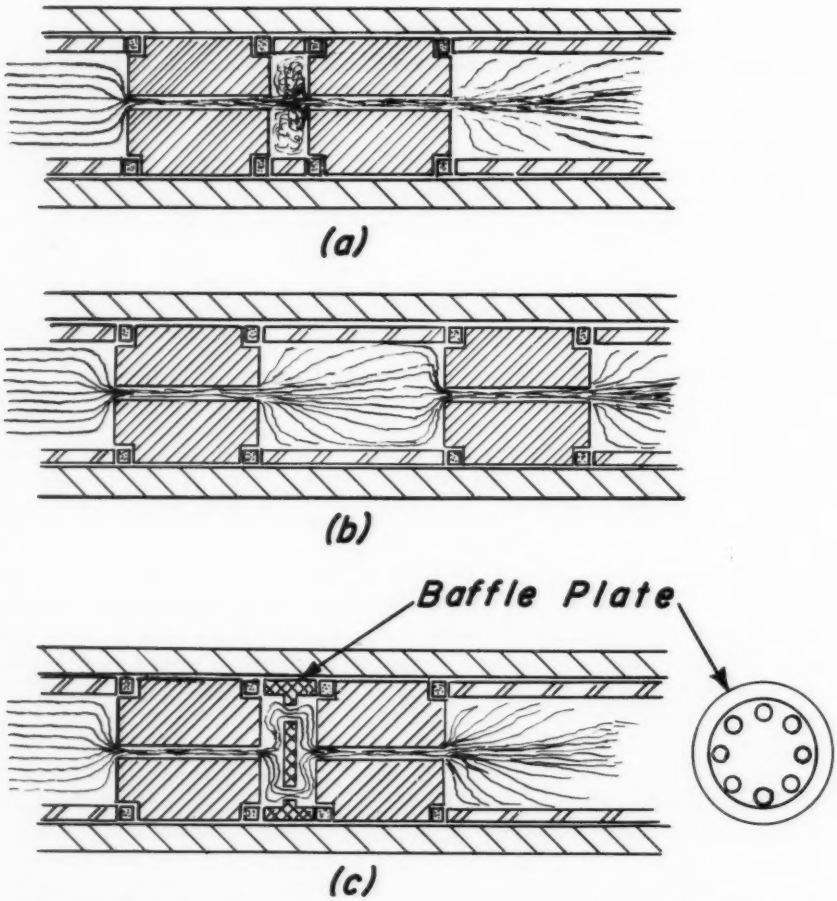

FIG. 2. Flow pattern in multi-stage valve arrangement: (a) short distance between valves; (b) long distance between valves; (c) baffle plate between valves.

TABLE 3

Effect of distance between valve exit and baffle surface on the efficiency of homogenization

Flow rate—300 gal/hr 0.6-in. orifice		
Distance (Valve exit to baffle)	Pressure drop	EHI [a]
(in.)	*(lb/sq in.)*	
0.01	1,525	44.6
0.1	1,525	52.6
0.16	1,525	61.5
0.29	1,525	61.5
0.63	1,525	68.0
2.44	1,525	82.1
Baffle not used	1,525	118.0

[a] EHI—Efficiency of homogenization index; inversely related to efficiency.

valves is increased (Figure 2b), the degree of expansion of the milk increases until a condition of fully developed flow exists in the intermediate space, resulting in full expansion and contraction losses for each of the two valves.

Since the distance that could be obtained between the valves was limited by the dimensions of the experimental valve body, baffle plates as shown in Figure 2c were installed between the valves to obtain fully expanded flow. A limited number of trials (Table 4) indicated that the introduction of a baffle plate between two valves in series did bring the total pressure drop into the theoretical range. Preliminary trials revealed that a baffle plate alone caused neither an appreciable pressure drop nor measurable homogenization. The hydraulic results of this experiment seem to support the suggested flow patterns.

DISCUSSION OF RESULTS

A different approach to gain evidence as to the mechanism of homogenization, using a specially designed short-tube type of valve, was taken in order to separate and control accurately the physical conditions normally associated with valve design.

The total pressure drop in a homogenizer valve is made up of friction, contraction, and expansion losses. The lack of change in pressure drop with increasing valve length would appear to indicate that the frictional loss in the experimental valve was negligible. This suggests that fully developed expansion probably did not occur in these valves. This view is supported by calculations of coefficients of discharge ($C = \frac{Q}{a\sqrt{2gh}}$ where Q = discharge in cubic feet per second, a = area in square feet, g = acceleration due to gravity, and h = head in feet), which ranged from 0.51 to 0.65. These coefficients would be in the range expected if the values were acting as sharp-edged orifices instead of short-tube valves (2).

Since the valve length was unrelated to homogenization efficiency, the factors of surface area and time of passage, which would be altered by changes

in valve length, could not be associated with homogenization efficiency within the range investigated. This is in contrast to the findings of Wittig (17), who presented photographs to indicate that the degree of homogenization was reduced as the time of passage was shortened.

It has been suggested by Wittig (15-17) that the first stage in homogenization involves an elongation of the milk fat globule to an extent varying with the clearance velocity. Although Wittig did not specify where this elongation takes place, it seems reasonable to assume that the sudden change from a comparatively static state immediately before the valve entrance to a region of high velocity within the valve clearance would cause elongation of the fat globule. The fact that the beveled valve decreased both the pressure drop and the homogenization efficiency permits the conclusion that homogenization is dependent in part upon the valve entrance conditions.

TABLE 4

Effect of series valve arrangement on pressure drop and the efficiency of homogenization

Valve arrangement			
Orifice length	Distance [a]	Pressure drop	EHI [b]
(in.)	*(in.)*	*(lb/sq in.)*	
0.2 } 0.3 }	0.06	1,500	136.1
0.2 } 0.3 }	0.19	1,450	102.8
0.2 } 0.3 }	2.44	2,250	90.3
0.2 } 0.3 }	Baffle	2,541	61.6

[a] Distance between valves in series.
[b] EHI—Efficiency of homogenization index; inversely related to efficiency.

The increase in efficiency of homogenization when baffle plates were used may be explained either by impact or by the intensity of turbulence. Wenrich (13) conducted experiments which indicated that as the distance from the valve entrance to the impact surface was increased beyond 3/32nds of an inch the effect of impact decreased rapidly. Since the results presented in this study indicate that considerable improvement in the efficiency of homogenization was obtained when the baffle plate was $2\frac{7}{16}$-in. from the valve exit, it appears inconceivable that impact could be entirely responsible for this improvement. It was thought that as the distance between the valve exit and the baffle plate was shortened, the turbulence resulting from the sudden expansion became more violent due to the confined area, and that the more radical change of direction increased the chaotic motion. The improvement in homogenization efficiency obtained at a constant pressure upon adding baffle plates shows that increased homogenization efficiency can be obtained without an increase in pressure drop.

There is some disagreement among investigators as to whether a given total pressure will produce more efficient homogenization when applied to a single valve or when divided among several valves in series (7, 16). The hydraulic results obtained in this study indicated that a relatively large volume was required between valves placed in a series to obtain an additive pressure drop. It was found necessary to use baffle plates between the successive stages to obtain a complete expansion and contraction. This indicates that the multi-stage homogenization, described by some investigators (6, 14, 16, 17), was not multi-stage in the sense of complete expansion and contraction losses at each stage. The use of fixed orifice valves in the present study made this effect more apparent than with the variable clearance valves used by the other investigators.

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METHOD FOR REMOVING CATIONIC RADIONUCLIDES FROM MILK

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SUMMARY

A new method for the removal of radioactive strontium, barium, and cesium from milk has been developed. Fresh samples of raw whole milk with added Sr^{85}, Ba^{140}, or Cs^{134} were stored at 4° C. for at least 72 hr. The samples were then adjusted to a pH of 5.4 or 5.3 with 0.1 or 0.5 *M* citric acid and passed through a column containing Dowex 50W-X8 cationic resin in the CaMgKNa cycle. The resin was previously charged with a mixed salt solution containing Ca, Mg, K, and Na chlorides in approximately the same relative proportions as in the milk, but at five times their usual concentrations, and at the same pH of acidified milk. After treatment, the milk was mixed with Dowex 2-X8 anionic resin in the OH^- cycle, by the batch process, and the pH was brought to 6.6. The milk was filtered through a cheese cloth to remove the spent resin. Analysis of treated milk indicated that approximately 90-95% of Sr^{85}, 85-95% of Ba^{140}, and 75% of Cs^{134} were removed by this treatment from 25 resin-bed volumes of milk. The removal of radionuclides did not vary with flow rates of 5-25 ml. per minute and the cationic composition of the milk was essentially unchanged. Although the milk was not coagulable by rennin, addition of 2-3 ml. of 1 *M* $CaCl_2$ solution per liter of milk yielded coagulum comparable to that obtained with untreated milk. Further treatment of the final product with a CaMgKNa resin at pH 6.6 resulted in an additional removal of approximately 45, 30, and 90% of residual Sr^{85}, Ba^{140}, and Cs^{134} remaining after the initial treatment without further change in the gross cationic composition of the milk. Results obtained at pH 5.2-5.3 with milk labeled in vivo and in vitro were similar.

Since the advent of nuclear weapons testing, the radionuclide contamination of foods has been of concern. Surveillance studies have indicated significant amounts of biologically important radionuclides such as strontium[89] (Sr^{89}), strontium[90] (Sr^{90}), iodine[131] (I^{131}), barium[140] (Ba^{140}), and cesium[137] (Cs^{137}) in milk (2). Because of the health hazards associated with some of these contaminants, particularly Sr^{90}, methods have been sought to minimize their levels in marketed dairy products.

This problem may be approached either at the point of production on the dairy farm (6, 11) or at the processing plants before the product reaches the consumer. Control at the dairy farm may require modification of farm and animal husbandry practices. Although this approach might accomplish an over-all reduction in the concentration of radionuclides thus far observed in milk, it would not be effective in an emergency situation involving higher exposures. Under such circumstances, treatment of milk would be necessary to reduce contamination to safe levels. This treatment must be simple, economical,

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and highly effective in removing the radionuclides in question and should be compatible with the practices and equipment employed in the milk plant. The treated product would be required also to meet established criteria of quality, including those relating to mineral balance, physical stability, palatability, and sanitation.

Several laboratory studies have been made on the removal of radioactive strontium, cesium, and iodine from milks labeled in vitro and in vivo. Ion exchange techniques using commercially produced synthetic ion exchange resins or naturally occurring materials such as bone preparations and calcium phosphate have been applied.

Pulverized inorganic bone preparations were found to remove 50-70% of Sr^{85} from in vitro labeled milk, but the treatment decreased the calcium content appreciably (12). Synthetic cation exchange resins in the Na^+, K^+, or Ca^{++} forms have been used extensively. Evidence has been presented to show the effectiveness of these resins in the removal of radiostrontium (3-5, 7, 10) and radiocesium (7, 8) from milks labeled in vitro and in vivo. Both batch and column techniques have been employed. The removal of up to 90% of strontium and cesium was always accompanied by gross changes in the cationic composition of the milk (4, 5, 10, 12) and in some of its physical and chemical properties such as pH, titrable acidity, curd tension, and rennin coagulation time (4). Removal of radionuclides from milk labeled in vitro was always higher than from milk labeled in vivo. Milk equilibrated [2] with added radionuclides for at least 72 hr. in vitro gave results comparable to those obtained with milk labeled in vivo (8).

To maintain the cationic composition of milk unaltered, a resin charged with a synthetic mixed salt solution simulating the major cations in milk was found necessary. Although this technique preserved the cationic composition essentially unchanged, the removal of strontium was only 66% and of cesium 85%. To remove higher percentages of strontium a shift in equilibrium was accomplished in which the colloidally bound divalent cations were removed by use of pairs of CaKNa resins. In the first treatment Ca and Sr both were removed; in the second, the resin gave up Ca and removed excess Na from milk. This procedure removed approximately 85% of the strontium content (8). Although this careful manipulation of cationic composition of milk to remove Sr is possible by the batch process, its suitability for column techniques, especially to handle large volumes of milk, is questionable.

From the foregoing observations it is evident that the removal of radionuclides from milk can be achieved only if the bound ions are transformed into more readily exchangeable forms. The physico-chemical nature of milk is such that alteration of its pH from 6.6 to 5.2 will shift the equilibria so that the colloidally bound ions are released to become free ionic species. Thus, passing acidified milk through a suitable cation exchange resin charged with synthetic

[2] The term equilibrate refers to the procedure whereby milk with added tracer is allowed to stand for a given length of time to arrive at a condition of equilibrium between the tracer and other constituents of milk.

mixed salt solution under appropriate conditions should result in the removal of a high percentage of radionuclides without any significant change in cationic composition. Neutralization of acidified and resin-treated milk to pH 6.6 with an anionic resin in the hydroxyl cycle should restore some of the original physical and chemical properties, resulting in an acceptable product.

An investigation of these possibilities has resulted in the method described here.

EXPERIMENTAL PROCEDURE

Materials. Milk labeled in vitro was prepared by adding an aliquot of a Sr^{85}, Cs^{134}, or Ba^{140} solution of known activity to milk and allowing the mixture to equilibrate at 4° C. for at least 72 hr. before use. Milk labeled in vivo was obtained from goats dosed orally with approximately 85 μc. of Sr^{85} and 200 μc. of Ca^{45} and was obtained from the Laboratory of Radiation Biology, Department of Physical Biology, Cornell University, Ithaca.

The cation exchange resin used was Dowex 50W-X8 (H^+), 20-50-mesh size, with a total exchange capacity of approximately 2.0 meq/ml of resin in the wet form.

The anion exchange resin used was Dowex 2-X8 (Cl^-), 20-50-mesh size, and having a total exchange capacity of approximately 1.33 meq/ml of wet resin.

Resin regeneration procedure. Since the Dowex 50W-X8 resin, as purchased, was in the hydrogen form, it was necessary to regenerate it on a calcium, potassium, and sodium (CaKNa) or a calcium, magnesium, potassium, and sodium (CaMgKNa) cycle. The regeneration of the resin was performed as follows: First, the cationic composition of milk was determined according to the rapid technique of Murthy and Whitney (9). A mixed salt solution was prepared to contain Ca, Mg, K, and Na in the same relative proportions as in the milk but in five times the concentration, and the pH was adjusted to be the same as that of the acidified milk, with 0.1 *M* HCl. The solution was passed down-flow through a glass column (2.3 by 20 cm.) containing 30 g. of the resin, at approximately 4-5 ml. per minute, until the pH and composition of the effluent were the same as of the influent. After this equilibrium level was reached, excess salts were removed by washing the resin with distilled water.

The anion exchange resin was converted from the chloride to the hydroxyl form by treating the resin with 5% NaOH. After the resin was fully charged, excess NaOH was removed by washing the resin with distilled water.

Procedure. Two and one-half liters of milk equilibrated with added radioactive tracer was placed in a three-liter beaker. Citric acid solution (0.1 or 0.5 *M*) was added from a burette and the milk was stirred constantly with a mechanical stirrer until the pH reached 5.4 or 5.3. The milk was transferred to a suitable container from which it was allowed to flow through the exchange column containing 30 g. of Dowex 50W-X8 cationic resin in the CaMgKNa cycle, at 10-15 ml. per minute. The milk was collected in a beaker and 5-g. portions of Dowex 2-X8 anionic resin in the OH^- cycle were added with stirring until the pH reached 6.6. The milk was filtered through a cheese cloth to remove the spent resin.

In the experiments concerning the removal of radiocesium, the neutralized milk was passed through a second column containing CaMgKNa resin at pH 6.6.

Analytical methods. Throughout this investigation the following analytical methods were employed:

1. pH was determined using a Beckman Model Zeromatic pH meter.
2. Calcium, magnesium, potassium, and sodium were determined by flame spectrophotometry, similar to the technique of Murthy and Whitney (9).
3. Radioactivity was determined by gross γ-counting or by gamma spectroscopy according to a method described by Branson *et al.* (1).
4. Rennin coagulation tests were performed as follows: To 50 ml. of milk maintained at $40.0 \pm 0.1°$ C. in a water bath, was added 2 ml. of a dilute rennin solution (1 ml. of commercial rennet extract diluted to 50 ml. with distilled water) and mixed well. The time elapsing between the introduction of rennin and the first visual signs of coagulation was taken as the coagulation time.
5. Flavor score was determined after pasteurizing milk samples at 145° F. for 30 min., using a slightly modified American Dairy Science Association scoring guide (13).

RESULTS AND DISCUSSION

Resin regeneration characteristics. An ideal system for charging the resin is probably the milk itself, because this would eliminate all the possible errors inherent in the preparation of synthetic salt solution. To determine what kind of results can be obtained under such conditions for the removal of radionuclides, 30 g. of Dowex 50W-X8 (Na^+) resin placed in a column was charged with 1.5 liters of milk at pH 6.6. Excess milk was removed by washing the resin with distilled water. Another portion of the same milk, previously equilibrated with Sr^{85}, was passed through the resin column. Various fractions of the effluent milk were analyzed for Sr^{85} activity and cationic composition. From results presented in Table 1 it can be seen that the cationic composition of the original and the resin-treated milks are not significantly different. A constant

TABLE 1

Effect of CaMgKNa resin treatment on the cationic composition of milk and the removal of Sr^{85} and Cs^{134} (resin charged with milk at pH 6.6)

Sample	Ca	Mg	K	Na	Removal of Sr^{85}	Removal of Cs^{134}
	(g/liter)				*(%)*	
Untreated	1.20	0.19	1.39	0.47	....	....
Treated						
1st 100-ml. effluent	1.17	0.16	1.41	0.47	52	100
4th 100-ml. effluent	1.18	0.17	1.44	0.48	50	96
8th 100-ml. effluent	1.19	0.16	1.45	0.49	47	71
12th 100-ml. effluent	1.20	0.18	1.45	0.49	45	37
16th 100-ml. effluent	1.21	0.20	1.41	0.48	41	6
20th 100-ml. effluent	1.16	0.18	1.43	0.48	39	3
24th 100-ml. effluent	1.18	0.18	1.44	0.50	36	0
28th 100-ml. effluent	1.17	0.17	1.42	0.47	33	0
Average (treated)	1.18	0.17	1.43	0.49		

removal of approximately 45-50% of Sr^{85} was achieved from treated milk up to 30 resin-bed volumes. These experiments demonstrate the feasibility of using resin treatment for the removal of radionuclides without significant alteration in the concentration of the major cationic composition. It is clear, however, that this method of charging has no practical applicaion. Exploratory studies were conducted to determine the composition and concentration of salt solutions for charging resins. Fifty-gram portions of Dowex 50W-X8 (H^+) resin were charged with 5 × and 10 × the concentration of Ca, Mg, K, and Na (as chlorides) present in milk, as described under Resin Regeneration Procedure, until the pH and the cationic composition of the influent and the effluent were similar. Results indicated that approximately 1,100 ml. of 5× or 950 ml. of 10× CaMgKNa solution was required to equilibrate the resin. When 10× CaMgKNa solution was used for charging, monovalent cations were preferentially adsorbed on the resin, whereas with dilute solutions the observed preference follows as Ca > Mg > K > Na. However, with 5× CaMgKNa solution, the equilibrium condition achieved was such that passage through the column of 1× CaMgKNa solution similar to that of milk showed no significant changes in the cationic composition of the effluent solution.

To determine the effect of treating milk with resins charged as above on the cationic composition and the removal of radionuclides from milk, portions of milk equilibrated with Sr^{85} were treated with the resin as described under Procedure. Results showed that while a constant removal of 90-95% of Sr^{85} was obtained from both the columns up to 25 resin-bed volumes of milk, the cationic composition of milk (Table 2) from 10 ×-CaMgKNa resin column was significantly altered, whereas the cationic composition of milk from 5 ×-CaMgKNa resin column remained essentially the same. Therefore, 5 ×-CaMgKNa salt solutions were employed for charging resins in all subsequent experiments.

TABLE 2

Effect of 5 ×-CaMgKNa and 10 ×-CaMgKNa resin treatments on the cationic composition of milk (resin charged with synthetic salt solution at pH 5.4)

Concentration of charging solution	Sample	Ca	Mg	K	Na
		(g/liter)			
	Untreated	1.15	0.13	1.36	0.50
	Treated				
5×	1st 100-ml. effluent	1.14	0.16	1.36	0.48
	4th 100-ml. effluent	1.13	0.13	1.38	0.48
	8th 100-ml. effluent	1.14	0.14	1.38	0.50
	12th 100-ml. effluent	1.12	0.15	1.40	0.49
	Composite	1.14	0.14	1.40	0.49
	Untreated	1.14	0.19	1.46	0.50
	Treated				
10×	1st 100-ml. effluent	0.94	0.15	1.52	0.54
	4th 100-ml. effluent	1.00	0.20	1.59	0.49
	8th 100-ml. effluent	1.06	0.24	1.48	0.51
	12th 100-ml. effluent	1.08	0.23	1.44	0.50
	Composite	1.02	0.20	1.51	0.52

Effect of flow rates on the removal of Sr^{85}. Commercial processing requires handling of large volumes of milk and, therefore, the evaluation of any strontium removal method must take this point into consideration. To obtain information on capacity or speed of the resin method, portions of milk equilibrated with Sr^{85} for 24 hr. and acidified to pH 5.3 were passed through columns containing 30 g. of CaMgKNa resin at flow rates of 5, 10, 15, 20, 30, and 35 ml. per minute, respectively. When analyzed for radioactivity, the corresponding effluents indicated Sr^{85} removals 97, 96, 97, 96, 95, and 91%. This suggested that increase of flow rate from 5 to 25 ml. per minute did not seriously reduce the removal of radionuclides from milk in the test columns, and that with larger columns a higher flow rate might be used.

Effect of acidification of milk on the removal of radionuclides. The divalent cations such as calcium, magnesium, strontium, and barium in milk are distributed between the serum and colloidal phases. That portion of salts bound with the colloids is not readily available for ion exchange. Acidification of milk ionizes colloidally bound salts which then become available for exchange. For acidifying milk, a suitable nontoxic acid is required. Since citric acid is a natural constituent of milk, and is used as a food additive, it was selected for this purpose. To determine the effect of acidification of milk on the removal of Sr^{85}, Ba^{140}, and Cs^{134}, 2-gal. lots of raw whole milk were equilibrated with added Sr^{85}, Ba^{140}, or Cs^{134}. Each lot was divided into several samples, adjusted to pH values ranging from 5.2 to 6.6 as described under Procedure, and passed through columns containing CaMgKNa resin at the same pH as the respective acidified milk sample. The 1st, 4th, 8th, 12th, etc., 100-ml. aliquots of effluents were collected and analyzed for radioactivity by gross γ-counting or by γ-spectroscopy. To determine the Ba^{140} activity, the various samples were stored at 4° C. for 2 wk. to achieve transient equilibrium between Ba^{140} and its daughter La^{140}. From the gamma spectra the concentrations of Sr^{85}, Cs^{134}, and Ba^{140} were computed from counting rates at the 510, 790, and 1,600 Kev. photopeaks, respectively. Results as presented in Figures 1 and 2 show that removal of Sr^{85} and Ba^{140} are affected by the degree of acidification, the removal dropping from approximately 98 to 53% of Sr^{85}, and 94 to 23% of Ba^{140}, as pH was increased from 5.2 to 6.6. Removal of more Sr^{85} than Ba^{140} at any given pH suggests a more tenacious binding of Ba than of Sr in the colloidal-protein phases of milk. To remove a high percentage of divalent cationic radionuclides, apparently it is necessary to reduce the pH of milk to 5.3-5.4. When lowered to 5.2 the pH becomes critical to protein stability, although the ions are in the dissolved state.

Removal of Cs^{134} was not affected by acidification of milk. This is to be expected, since monovalent cations are in the free ionic state in normal milk. However, the Cs^{134} was not removed as effectively as Sr^{85} or Ba^{140}, probably because of preferential adsorption of divalent rather than monovalent cationic radionuclides.

Comparison of Sr^{85} removal from milk labeled in vivo and in vitro. Because of the differences observed by other investigators in removal of radioactive

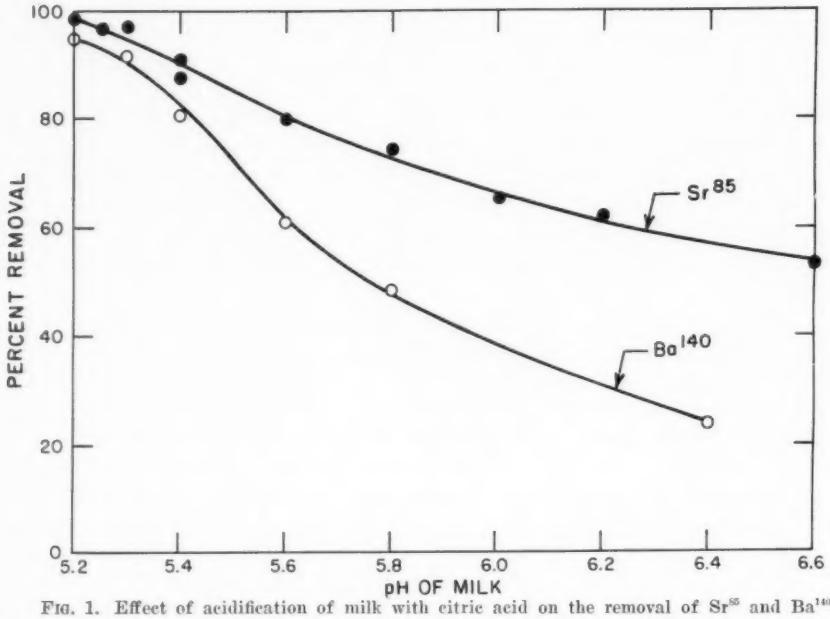

FIG. 1. Effect of acidification of milk with citric acid on the removal of Sr^{85} and Ba^{140} from milk.

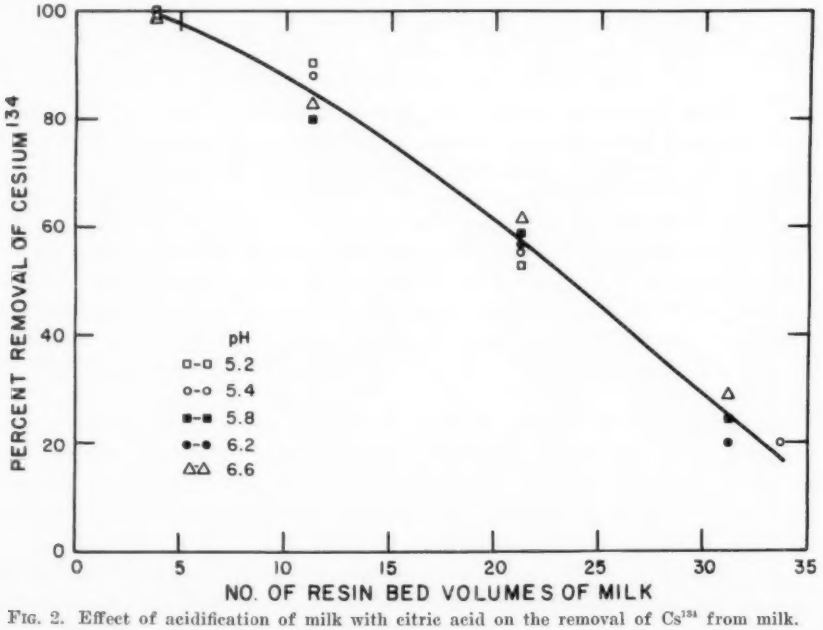

FIG. 2. Effect of acidification of milk with citric acid on the removal of Cs^{134} from milk.

strontium from milk labeled in vivo and in vitro, it was considered important to establish whether any significant differences existed between the milk samples thus labeled, especially when processed at pH 5.2-5.4. For this purpose, samples of goats milk labeled in vitro and in vivo were employed. The milk samples were divided into several portions, adjusted to pH values of 6.5, 6.2, 5.8, 5.4, and 5.25, as described before, and treated with cationic resin in the CaKNa cycle, charged at the same respective pH values. Results presented in Figure 3 show that at pH 5.2-5.3 approximately 93% of Sr^{85} was removed from both types of labeled milk, whereas at higher pH values the amount of Sr^{85} removed differed significantly with the labeling method. At normal pH of milk (6.65), a difference of approximately 12% was observed, which is in conformity with the published results (14). Therefore, it is reasonable to assume that the results obtained with properly equilibrated milk labeled in vitro, at pH of 5.2-5.3, will be essentially the same as those to be expected with milk labeled in vivo. These limitations of pH apply to removal of colloidally bound radionuclides. For removal of monovalent cationic radionuclides such as cesium, which occur in a free ionic state, results obtained with milk labeled in vitro and in vivo should be comparable at all the pH values studied herein.

Effect of number of resin-bed volumes of milk passed through the column on removal of radionuclides. To determine the volume of milk that could be treated with a given amount of resin for high percentage removal of radionuclides from milk at a constant rate, several trials were made similar to the technique de-

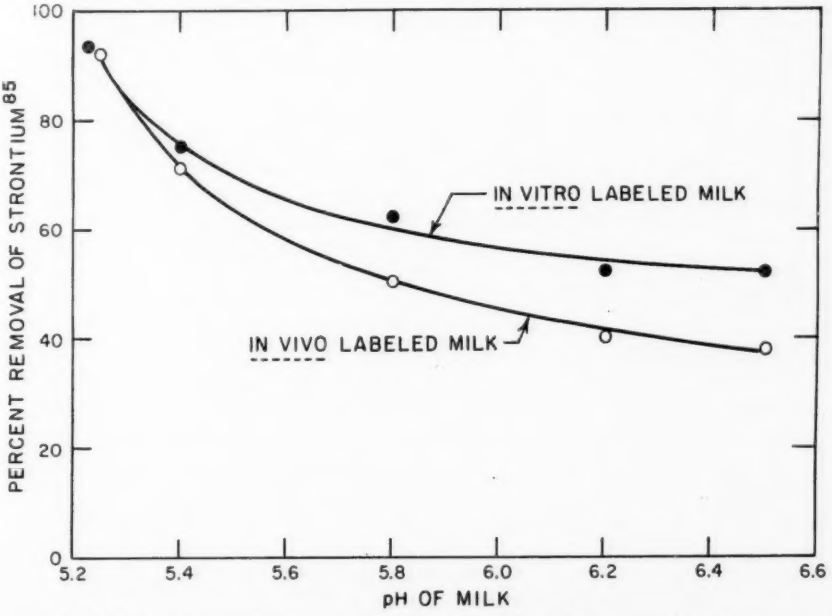

FIG. 3. Effect of pH on the removal of Sr^{85} from milks labeled in vivo and in vitro.

scribed before, using Sr^{85}, Ba^{140}, and Cs^{134}. Results are presented in Figure 4. These data show a constant removal of $94 \pm 2\%$ of Sr^{85} and $92 \pm 2\%$ of Ba^{140} up to 30 resin-bed volumes of milk, beyond which a gradual decrease was observed. The behavior of Cs^{134} is quite striking, in that its removal decreased from 98 to 0% with increase in the number of resin-bed volumes of milk from 5 to 50, revealing that this procedure was unsuitable for the removal of radiocesium.

Modification of the procedure for the removal of Cs^{134}. Since the previously described procedures for the removal of Sr^{85} and Ba^{140} were not satisfactory for the removal of radiocesium, certain modifications of the techniques were investigated. The hypothesis was advanced that when citric acid and resin treated milk is neutralized to pH 6.6 with an OH^- cycle anion exchange resin, the concentrations of Ca^{++}, Mg^{++}, Sr^{++}, and Ba^{++} in solution are reduced below that in the untreated milk by the complexing effect of these ions with the colloids and excess citrates. On the other hand, the monovalent cations such as Na^+, K^+, and Cs^+ do not form any such complexes and, therefore, would be present as free ions readily available to exchange reactions. To obtain information in this regard, milk equilibrated with added Cs^{134} was processed as described under Procedure, then passed through a second column containing CaMgKNa resin at pH 6.6. Results are presented (Figure 5). From 100 to

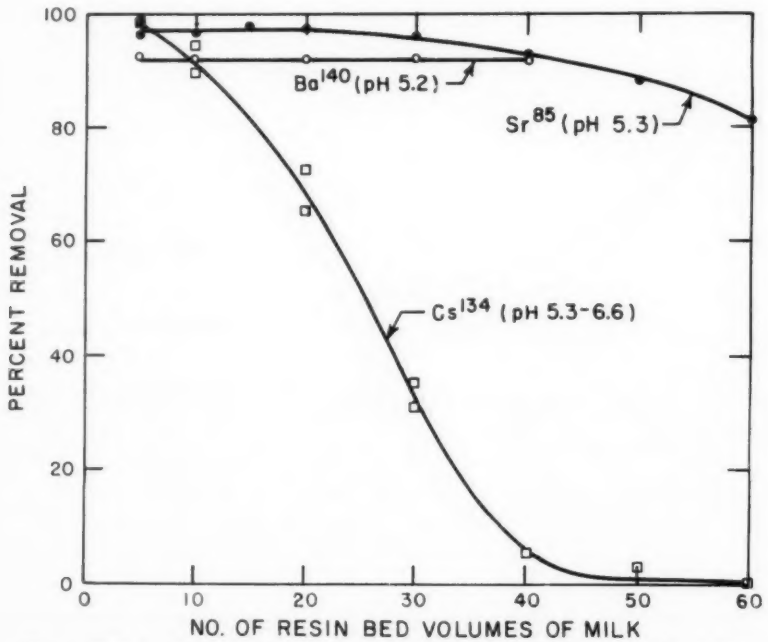

FIG. 4. Effect of number of resin bed volumes of milk passed through the column on the removal of Sr^{85}, Ba^{140}, and Cs^{134} from milk.

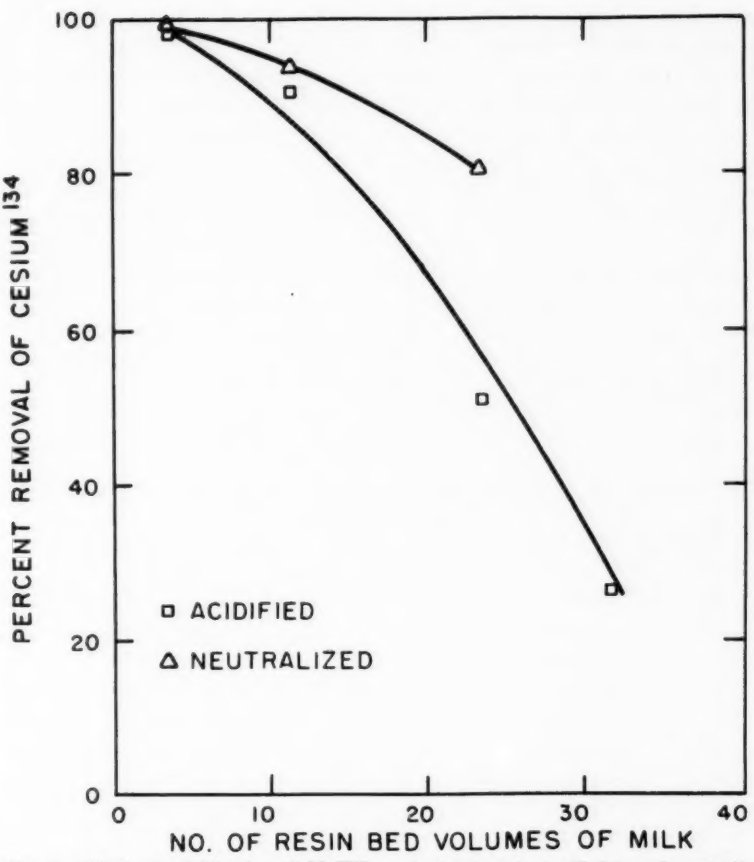

FIG. 5. Effect of acidification, CaMgKNa resin treatment at pH 5.3, and neutralization to pH 6.6, CaMgKNa resin treatment at pH 6.6 in succession on the removal of Cs^{134} from milk.

80%, with an average of 90%, of the residual Cs^{134} was removed by this neutralization and the second resin treatment.

As the pH of neutralization was increased above 6.6, more Cs^{134} was removed (99% at pH 7.7), indicating stabilization of the divalent cation complexes with citrates and colloids. However, pH values above 6.6 were considered unsatisfactory from the point of acceptability of the final finished product. This treatment also removed approximately 45 and 30% of the residual Sr^{85} and Ba^{140} at pH 6.6 and, therefore, suggests that neutralization to pH 6.6 does not completely reduce the concentration of divalent cations to be unavailable for ion exchange reactions. Since more Cs^{134} was removable by this neutralization and second resin treatment than from unacidified milk at pH 6.6, it makes the hypothesis valid up to a certain extent only. The over-all effect of these two-

column resin treatments resulted in the removal of 95-98% of Sr^{85}, 90-95% of Ba^{140}, and 95-100% of Cs^{134}.

Cationic composition of milk. Untreated milk and milk treated with cationic and anionic resin, as employed in some of the experiments described before, were analyzed for the major cations. The results are presented in Tables 3 and 4, which show that the Ca, Mg, K, and Na content is essentially the same in the untreated and treated milks. This indicates that the method described herein may be successfully employed for the removal of cationic radionuclides from milk.

Rennet coagulation studies. Several fresh samples of raw whole milk were acidified, resin-treated, and neutralized as described before. When rennet solution was added to portions of these samples, no coagulation was observed. This is to be expected because of the complexing effect of citrates with calcium, thus making calcium unavailable for the formation of coagulum. This is evident:

TABLE 3

Effect of CaMgKNa resin treatment at pH 5.4 and neutralization to pH 6.6 with OH^- resin on the cationic composition of milk

Sample	Treatment	Ca	Mg	K	Na
		(g/liter)			
1	Before	1.15	0.13	1.36	0.50
	After	1.14	0.14	1.38	0.49
2	Before	1.12	0.10	1.32	0.44
	After	1.12	0.10	1.28	0.44
3	Before	1.11	0.19	1.32	0.49
	After	1.12	0.17	1.36	0.50
4	Before	1.14	0.18	1.43	0.49
	After	1.14	0.15	1.42	0.48

TABLE 4

Effect of CaMgKNa resin treatment at pH 5.4, neutralization to pH 6.6, and CaMgKNa resin treatment at pH 6.6, in succession on the cationic composition of milk

Treatment	Sample	Ca	Mg	K	Na
		(g/liter)			
	Untreated	1.15	0.13	1.36	0.50
	Treated				
Column at pH 5.4	1st 100-ml. effluent	1.14	0.16	1.36	0.48
	4th 100-ml. effluent	1.13	0.13	1.38	0.48
	8th 100-ml. effluent	1.14	0.14	1.38	0.50
	12th 100-ml. effluent	1.12	0.15	1.40	0.49
	Composite	1.14	0.14	1.40	0.49
	Treated				
Column at pH 6.6	1st 100-ml. effluent	1.15	0.13	1.32	0.43
	4th 100-ml. effluent	1.14	0.15	1.31	0.43
	8th 100-ml. effluent	1.13	0.17	1.34	0.44
	Composite	1.14	0.15	1.32	0.43

that if the calcium is added to milk prior to rennet, near-normal coagulation will occur. Addition of 2-3 ml. of 1 *M* $CaCl_2$ per liter of milk yielded coagulum comparable to that obtained with untreated milk.

Organoleptic quality of resin-treated milk. Fresh raw whole milk was divided into several portions, acidified to different pH values, resin-treated, and neutralized as described before. The various samples were pasteurized at 145° F. for 30 min. and submitted to a taste panel. A portion of original milk served as a control. The judgments were evaluated by a ten-judge panel, using a slightly modified American Dairy Science Association scoring guide, and the averaged results are presented in Table 5. These limited data indicate that the

TABLE 5

Effect of resin treatment on the flavor score of milk

Initial pH	Cation resin treatment	Method of restoring pH	Average flavor score
5.4	No	Anionic resin	35.5
5.4	Yes	Anionic resin	36.0
5.8	No	Anionic resin	36.0
5.8	Yes	Anionic resin	36.0
6.2	No	Anionic resin	36.2
6.2	Yes	Anionic resin	35.2
6.68	No	None	36.6
6.68	Yes	None	36.1

organoleptic quality of milk treated by this process is comparable to that of normal pasteurized milk.

While these investigations with ion exchange resins have provided a method suitable for the removal of cationic radionuclides from milk, its applicability to commercial operations is yet to be assessed. Further work in regard to physico-chemical properties, bacteriological and nutritional qualities, and methods for sanitizing and stripping of resin columns needs to be done.

ACKNOWLEDGMENT

The authors are grateful to Dr. C. L. Comar of the Laboratory of Radiation Biology, Department of Physical Biology, Cornell University, Ithaca, for supplying goats' milk labeled in vivo; to Mr. Ulysses Rhea for his help in the analysis of milk samples for major cations; and to the members of the Taste Panel of the Dairy Products Division, USDA, Beltsville, Maryland, for their help in determining flavor quality of milk samples.

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SOME FACTORS AFFECTING THE HEATING AND COOLING LAGS OF PROCESSED CHEESE IN THERMAL DEATH TIME CANS[1, 2]

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SUMMARY

The lag correction factor, the time that must be subtracted from the gross heating time to give the net time at heating medium temperature, varies with the fill-weight and the position of the can for conduction heating of cheese spreads. Results of this study indicate that: (a) The lag correction factor increases with increases in the fill weight. (b) The lag correction factor was larger when the can was heated in the flat position as compared to the edge position.

Thermal death time (TDT) cans 2.5 in. in diameter and 0.375 in. deep, designated as 208 × 006 in canning industry nomenclature, are used as containers for food products for evaluating the effect of heat on microorganisms suspended in a food product. The experiment is usually designed so that the majority of the heating effect takes place at the temperature of the heating medium. However, since the TDT cans can not be instantly heated or cooled, it is necessary to correct the total heating time for lags in heating and cooling.

Several factors are known to affect the heating and cooling lags. Townsend *et al.* (4, 5) determined that the heating characteristics of TDT cans were affected by the quantity of product in the container and the physical characteristics of the product. Sognefest and Benjamin (3) reported that the position of the can during heating affected the rate of heat penetration when heating took place primarily by convection.

The studies reported herein were performed to determine the specific effect of fill-weight and can position on: (a) the heating characteristics of TDT cans of processed cheese spread, and (b) the subsequent lag correction factors.

EXPERIMENTAL METHODS

The usual practice when making heat effect studies at high temperatures using TDT cans is to start measuring heating time when steam is turned on and stop the moment the steam is turned off and the cooling water turned on; this total heating time is designated as B. The net heating time (U) in minutes at the heating medium temperature is the total heating time (B) minus the lag correction factor (t_c). The factor t_c includes the correction for the lag in heating and the lag in cooling.

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[1] Michigan State University Agricultural Experiment Station Journal No. 2730.

[2] This manuscript describes a portion of the research contained in a thesis prepared by the senior author in partial fulfillment of the requirements for the degree Doctor of Philosophy at Michigan State University.

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Copper-constantan thermocouples were mounted in 12 TDT cans at the point of slowest heating (Figure 1). Sognefest and Benjamin (3) found that

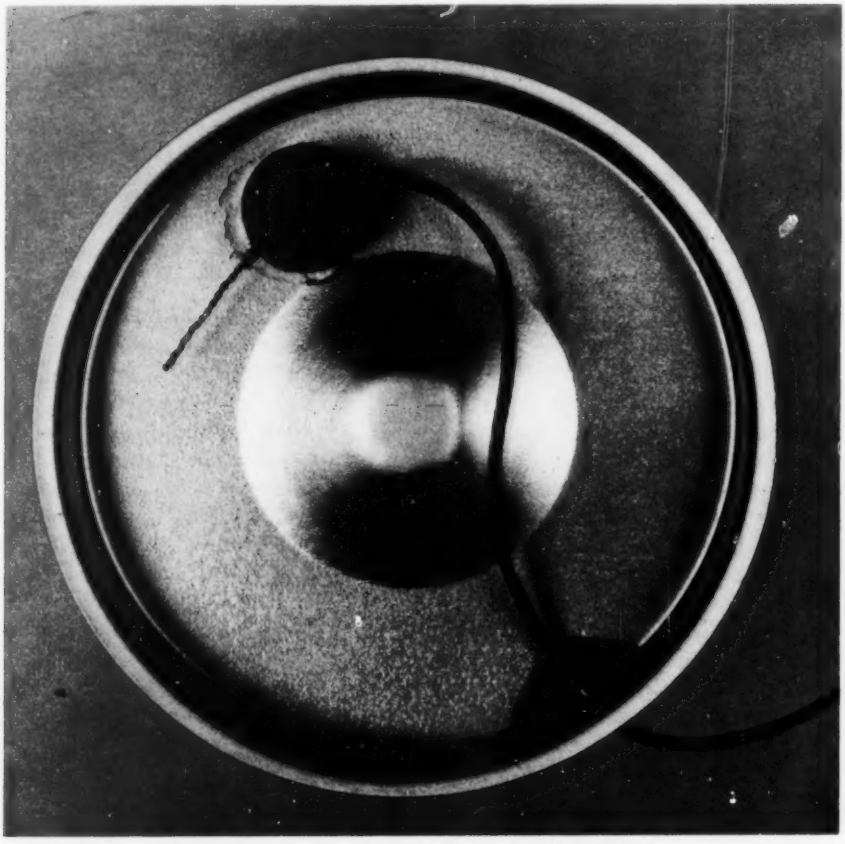

FIG. 1. TDT can and copper-constantan thermocouple.

the dimple in the bottom of the TDT cans displaced the slowest heating point from the geometrical center to a point located between the side and center midway between the top and bottom. The thermocouples were made from No. 30 B & S gauge wire and were held in position by passing the wire through a hole drilled in a section of a No. 00 rubber stopper. The lead wire was brought out through a hole punched in the lower side of the TDT can. An electrical insulating resin [4] was used to secure the stopper in place and seal the hole where the lead wire was brought out of the can.

The jig that supported the TDT can during the can-closing operation was

[4] Furane Plastics Inc., 4513 Brazil Street, Los Angeles 39, California.

notched so that the thermocouple lead wire did not interfere with the closing machine.

The cans were heated in a miniature retort similar to the one described by Townsend *et al.* (4). Cans were tested with fill-weights of 17, 19, and 21 g. (the maximum capacity of TDT cans is approximately 21 g.) of prepared processed cheese spread. The cans were heated flat and on edge. The heating and cooling curves were plotted and the f_h and j for each curve determined by the method described by Ball (1). An example of these determinations is presented in Figure 2.

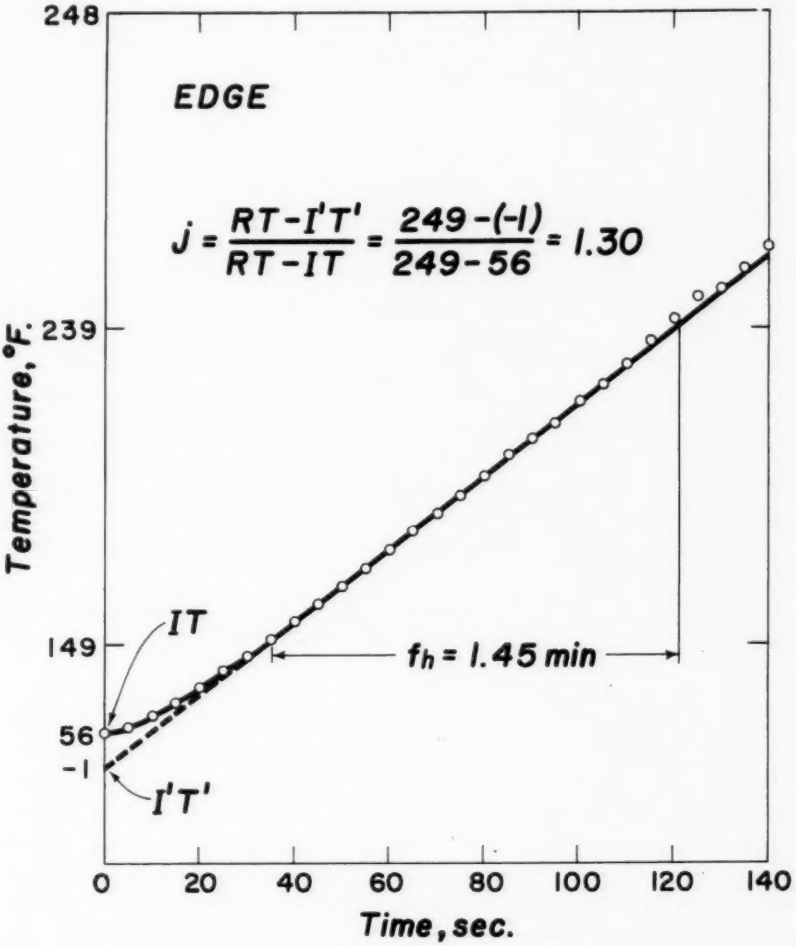

FIG. 2. A typical heat penetration curve, illustrating the method by which the f_h and j values are determined.

When the heating and cooling data may be approximated by straight lines when plotted on semilogarithmic paper (Figure 2), the lag correction factor can be calculated as a simplified thermal process calculation using the method of Ball (1). Using a z-value, which is a measure of the slope of the thermal destruction curve of the microorganism or chemical under study, of 18° F., and calculating the lag correction to 0.1° F. below heating medium temperature, the lag correction factor can be calculated by the following equation developed by Pflug and Esselen (2).

$$t_c = f_h \ (\log \ j \ I - 0.725)$$

where f_h = a measure of the slope of the heating curve
j = lag factor
RT = heating medium temperature
IT = initial temperature
$I = RT - IT$.

RESULTS AND DISCUSSION

The calculated lag correction factors for the different fill-weights and the different positions of the TDT cans are shown in Table 1. In the calculations

TABLE 1

Effect of fill-weight and can position on the lag correction factor of a processed cheese spread in TDT cans
(Average of two trials)

	Position of the cans					
	Edge			Flat		
Fill-wt.	f_h	j	t_c	f_h	j	t_c
(g.)	*(min.)*		*(min.)*	*(min.)*		*(min.)*
17	1.44	1.30	2.41	1.87	0.90	2.83
19	1.74	1.20	2.82	2.00	1.06	3.16
21	1.83	1.20	3.00	2.04	1.12	3.28

it was assumed that $I = RT - IT = 194°$ F. Since f_h and j do not change with changes in I, the lag correction factor for other values of I can be easily calculated.

Results show that each increase in the fill-weight resulted in an increased lag correction factor; the greater the fill weight, the slower the rate of heating and cooling (larger f_h) and, consequently, the larger the lag correction factor.

The f_h of the heating curve and, therefore, the lag correction factors were greater when the TDT can was in the flat position as contrasted to the edge position. Due to the configuration of the TDT can, less of the product would be in direct contact with the metal of the can when it is in the flat position than when it is on its edge, unless the can was completely full. Since less product is in contact with the heat transfer surface with the TDT can in the flat position, heating will be slower, resulting in a larger f_h and, therefore, a greater lag correction factor. The cans tested in the flat position had the closure side up;

because of the shape of the TDT can, tests performed with the closure side down might have given slightly different results.

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GAS PRODUCTION BY ASSOCIATED SWISS CHEESE BACTERIA[1]

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SUMMARY

Gas production in skimmilk cultures was markedly stimulated and inhibited by interactions of the late gas-formers with some but not all of the Swiss cheese lactic flora. The starter lactobacilli were markedly stimulatory for both *Clostridium tyrobutyricum* and *Propionibacterium shermanii*. *Streptococcus thermophilus* was very stimulatory for the clostridium but without effect on the propionic, whereas *Streptococcus faecalis* generally had little effect on either gas-former. The nisin-producing strain of *Streptococcus lactis* was markedly inhibitory for the clostridium but without much effect on the propionic, whereas the ordinary strain of *S. lactis* had little effect on either. Gas production was stimulated when the clostridium and propionic were grown together. The stimulatory and inhibitory properties of the skimmilk cultures for the late gas-formers were at least in part dialyzable, heat- and acid-stable substances. The stimulatory activity of *Lactobacillus helveticus* was shown to be correlated with metabolic products rather than with the number of cells in the lactic culture.

The appearance and numbers of eyes in Swiss-type cheese depend on the timing, rate, and duration of the gas-producing bacterial fermentations in the cheese mass (2). Normal eye formation has been attributed to the fermentation of lactate by members of the genus *Propionibacterium* (7, 13). Eyes rough in appearance, too large, and too numerous, as well as splitting and cracking of the cheese, are late gas defects resulting from the production of excessive amounts of gas after the curd has lost its elasticity late in the ripening process. Late gas defects have been associated with fermentation of lactate by butyric acid bacteria (1, 10) and propionic acid bacteria (6). Van Beynum and Pette (12) named the lactate-fermenting butyric anaerobes *Clostridium tyrobutyricum*.

In the manufacture of Swiss cheese a number of populations of lactic acid bacteria may have grown and declined by the time the gas-forming propionics and clostridia appear (3, 4). Swartling and Lindgren (9) reported that growth and acid formation of species of *Clostridium* were promoted when grown with coliforms, streptococci, leuconostocs, lactobacilli, propionics, and *Bacillus subtilis*. Swiss cheese starter bacteria found stimulatory included *Streptococcus thermophilus*, *Lactobacillus lactis*, and *Propionibacterium* spp. The present work was undertaken as an investigation of the interactions of the lactics, propionics, and clostridia found in Swiss-type cheese.

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MATERIALS AND METHODS

Gas-producing fermentations were followed by means of the gasometer shown in Figure 1. All fermentations were carried out in either fresh or reconstituted skimmilk from bulk sources. Gas evolved from the fermentation in Test Tube A (Figure 1) displaced an acid-salt solution (5% H_2SO_4, 20% Na_2SO_4) from the 10-ml. pipette, B, into the reservoir, C. Changes in the volume of gas were read on the pipette with the fluid in the pipette and reservoir at the same level. Readings were corrected for changes in temperature or pressure by comparison with a gasometer attached to an uninoculated control.

Portions of skimmilk were subjected to 48-hr. fermentations by the following lactic acid bacteria:

Lactic acid bacteria	Developed titratable acidity [a]
	(%)
Streptococcus thermophilus Strain MC	0.48
Lactobacillus bulgaricus Strain Gere A	1.77
Lactobacillus helveticus Strain H-80	2.12
Lactobacillus lactis Strain 39a	1.08
Streptococcus faecalis Strain DK	0.31
Streptococcus lactis Strain X-13 (nisin)	0.28
Streptococcus lactis Strain Rogers	0.31

[a] Titratable acidity of uninoculated control subtracted.

An uninoculated portion of the original skimmilk was refrigerated while the lactic fermentations were taking place. The first four organisms listed are commonly used Swiss cheese starter bacteria, whereas *Streptococcus faecalis* is a heat- and salt-tolerant milk organism that can grow at the temperature of cheese in the press. *Streptococcus lactis* is commonly present in raw milk delivered to cheese factories and may include nisin-producing strains (represented here by Strain X-13) such as have been used to inhibit butyric acid fermentations in cheese.

At the end of the incubation period, the developed titratable acidity of each culture was determined and sufficient DL-lactic acid was added to all samples, including the unfermented control, to bring the titratable acidity up to 2.5% and the pH down to about 3.5. The pH was adjusted back to 5.75 by the careful addition with mixing of a slurry of $Ca(OH)_2$. By this procedure, all cultures had some DL-lactic acid present and the total lactate concentration of all cultures was about equal. As soon as the pH was adjusted to 5.75 each culture was chilled in ice to stop further lactic fermentation. When the pH of all cultures and the control had been adjusted, they were sterilized by autoclaving at 120° C. for 15 min. The pH of each culture was checked following autoclaving and where necessary readjusted to pH 5.75 by the careful aseptic addition of $Ca(OH)_2$.

Secondary gas-forming fermentations by *Propionibacterium shermanii* 31C (from J. M. Sherman) and *Clostridium tyrobutyricum* S-12 (isolated from

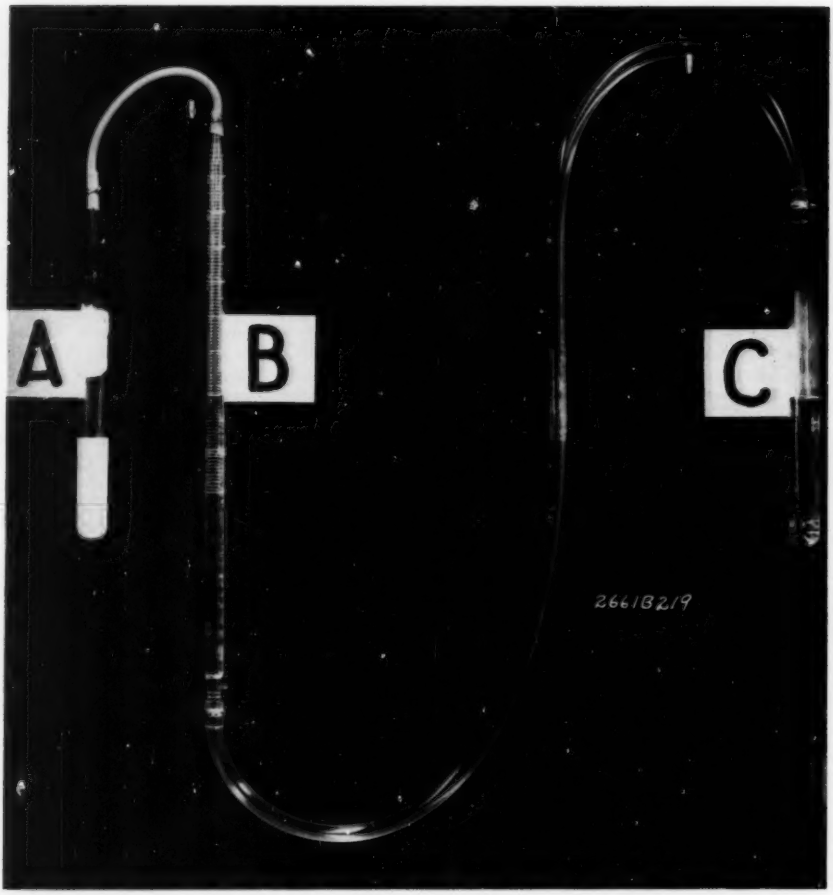

FIG. 1. Gasometer for test tube-sized fermentations. A. Fermentation tube. B. Ten-milliliter pipette containing displacement fluid. C. Displacement fluid reservoir.

Swiss cheese) were followed in each of the lactic fermented milks. The clostridial and propionic inocula for the secondary gas-forming fermentation were grown in APT broth, harvested while in the logarithmic phase of growth by centrifugation, and washed twice by resuspension in a pH 5.75 buffer containing $M/30$ KH_2PO_4, 0.1% peptone, and 0.05% sodium thioglycolate. Concentration of cells in the inoculum was adjusted so that 0.3 ml. contained 10^7 clostridium cells or 10^8 propionic cells, and this quantity was placed in the bottom of a sterile cotton-plugged test tube. Ten milliliters of the freshly autoclaved and cooled lactic milk cultures were immediately transferred by pipette to the previously labeled tube containing the inoculum. The cotton plugs in the culture tubes were clipped off, and the butt end was forced down into the neck of the

culture tube; the rubber stoppers bearing the hose connections were inserted and the stoppered tubes placed in a vacuum desiccator. The atmosphere was replaced with 90% N_2 and 10% CO_2 by evacuation and flushing (11). The gasometer headspaces were filled with nitrogen gas by raising the reservoir of displacement fluid until the entire pipette was filled and then forcing down the level of displacement fluid with a stream of high-purity nitrogen. The fermentation tubes were removed from the vacuum desiccator and attached to gasometers.

Secondary gas-forming fermentations were run in triplicate on each lactic milk culture. Cumulative measurements of gas evolution were made, and the arithmetic mean of the three replicates plotted for purposes of comparison of the length of lag periods and maximum rates of gas production. For purposes of statistical comparison the three replicates were plotted separately and individual values representing lag and maximum rate of gas production taken from the graphs. The lag period was defined as the time from inoculation required for gas production to become exponential. The largest amount of gas produced during any 10-hr. period was considered in calculating the maximum rate of gas production.

Dialysates were prepared by immersion of a cellulose dialysis tubing bag of distilled water into each lactic milk culture and milk control. Dialysis was allowed to proceed for three days in the refrigerator with frequent swirling. The bags were carefully withdrawn and rinsed with distilled water, and the contents were removed and used as the cell-free dialysates of the lactic cultures.

The statistical significance of differences in gas production was determined by means of the F test (8). The 0.05 level of probability was taken as statistically significant and the 0.01 level as highly significant.

RESULTS

Effect of initial lactic fermentation on subsequent gas-producing fermentations. Gas production by *Clostridium tyrobutyricum.* Gas production by *C. tyrobutyricum* in skimmilk fermented by various lactics was compared to gas production in skimmilk which had not undergone a lactic fermentation (Figure 2). The four Swiss cheese starter bacteria (*L. helveticus, L. bulgaricus, L. lactis,* and *S. thermophilus*) markedly stimulated gas production by the clostridium. *L. helveticus* and *L. bulgaricus* were particularly effective, both in shortening the lag and in increasing the rate of gas production.

Gas production by the clostridium was not significantly altered by *S. lactis* Strain Rogers or *S. faecalis,* with the exception of a 100-hr. lengthening of the lag period in the *S. faecalis*-fermented milk.

Skimmilk in which *S. lactis* X-13, a nisin-producing lactic strain, had grown was markedly inhibitory for the clostridium, lengthening the lag time appreciably and suppressing the gaseous fermentation.

Gas production by Propionibacterium shermanii. Portions of the lactic-fermented skimmilks prepared for the previous experiment were inoculated with 10^7 cells of *P. shermanii* per milliliter of milk. The procedures for inoculation

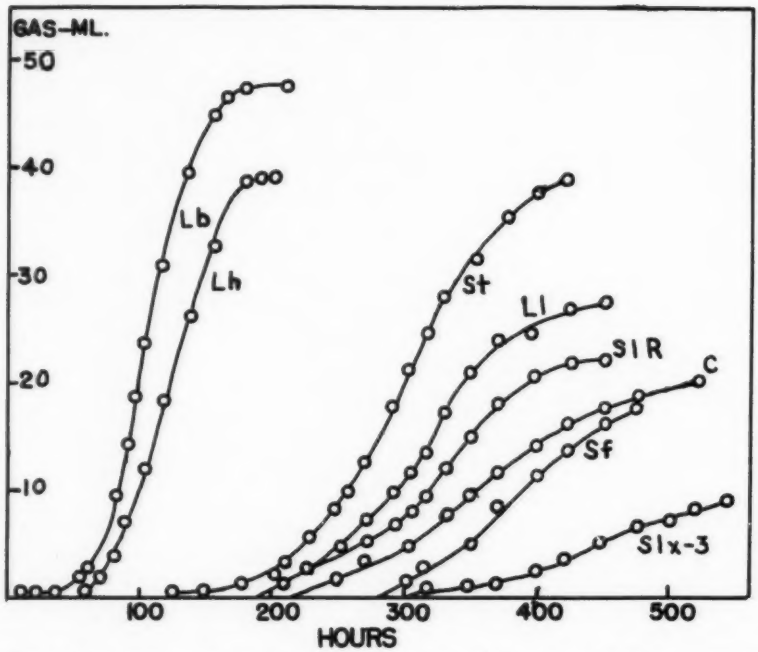

FIG. 2. Gas production by *C. tyrobutyricum* at 30° C. and pH 5.75 in reconstituted skimmilk fermented by various lactics. Key: Lb, *L. bulgaricus*, Lh, *L. helveticus*, St, *S. thermophilus*, Ll, *L. lactis*, SlR, *S. lactis* Strain Rogers, C, control, Sf, *S. faecalis*, SlX-13, *S. lactis* Strain X-13.

and gas measurement were the same as for *C. tyrobutyricum*. Of the various lactics employed only *L. bulgaricus* had a statistically significant effect on the maximum rate of gas production by the propionic (Figure 3). A significant shortening of lag in gas production by the propionic was also brought about by an initial fermentation by *L. bulgaricus* and, in addition, by *L. helveticus*, *S. lactis* Strain Rogers, and *S. lactis* X-13. There was no evidence of inhibition of the propionic by the nisin-producing *S. lactis* X-13.

Gas production by Clostridium tyrobutyricum and Propionibacterium shermanii in mixed fermentation. Mixed fermentations by *C. tyrobutyricum* and *P. shermanii* were run in portions of the same lactic milk cultures used in the preceding experiments. These portions were inoculated simultaneously with 10^6 cells of *C. tyrobutyricum* and 10^7 cells of *P. shermanii* per milliliter, and gas evolution was followed in the gasometer (Figure 1). Results are shown in Tables 1 and 2.

A consistent stimulation in gas production was noted in all of the mixed butyric-propionic fermentations, as compared to the separate fermentations. The degree of stimulation varied markedly, depending on which lactic had fermented the skimmilk initially. In fermented milk already quite favorable

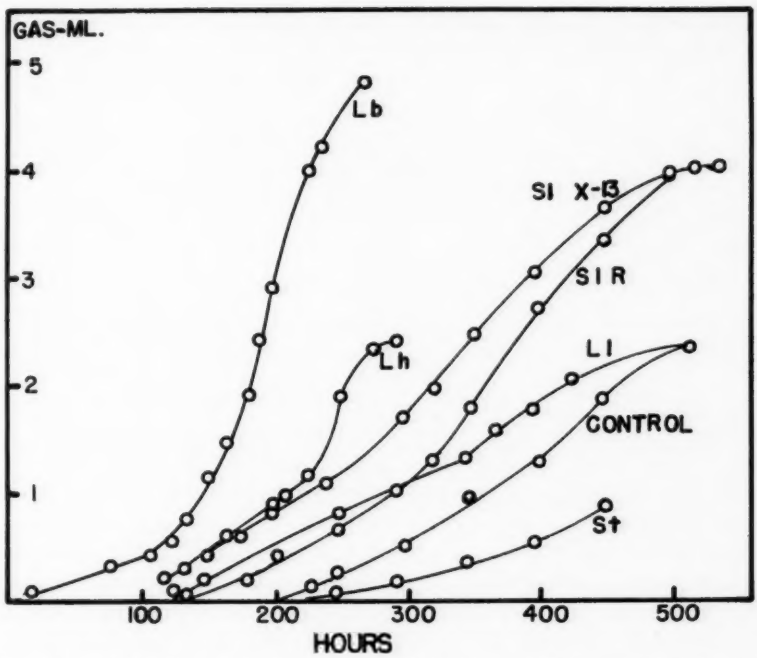

FIG. 3. Gas production by *P. shermanii* at 30° C. and pH 5.75 in reconstituted skimmilk fermented by various lactics. Key: Lb, *L. bulgaricus*, Lh, *L. helveticus*, SlX-13, *S. lactis* Strain X-13, SlR, *S. lactis* Strain Rogers, Ll, *L. lactis*, St, *S. thermophilus*.

TABLE 1

Effect of associative growth on lag in gas production at pH 5.75 and 30° C. by *C. tyrobutyricum* and *P. shermanii* in skimmilk fermented by various lactics [a]

Lactic organism	Length of lag time in initiation of gas production		
	C. tyrobutyricum	*C. tyrobutyricum* and *P. shermanii*	*P. shermanii*
	(hr.)		
L. helveticus	50**	47–*	123*
L. bulgaricus	35**	27–**	65*
L. lactis	187	120	120
S. thermophilus	127*	127	258
S. faecalis	282*	**– 94–**	285
S. lactis Strain Rogers	119	101	132*
S. lactis X-13	376**	105	114**
None—control	170	142–*	220

* Significant difference ($0.05 > P > 0.01$) from the control.
** Highly significant difference ($P < 0.01$) from the control.
**– Compared to *C. tyrobutyricum* fermentation of the same lactic fermented milk.
–** Compared to *P. shermanii* fermentation of the same lactic fermented milk.
[a] The lactic fermented milks were autoclaved prior to inoculation with the gas-formers.

TABLE 2

Effect of associative growth on maximum rate of gas production at pH 5.75 and 30° C. by *C. tyrobutyricum* and *P. shermanii* in skimmilk fermented by various lactics [a]

Lactic organism	Maximum rate of gas production		
	C. tyrobutyricum	*C. tyrobutyricum* and *P. shermanii*	*P. shermanii*
	(ml/10 hr.)		
L. helveticus	4.3**	7.4*	0.20
L. bulgaricus	4.0**	11.2*	0.49**
L. lactis	3.9**	7.1	0.10
S. thermophilus	2.6**	6.7**	N.D.
S. faecalis	1.1	5.5**	0.14
S. lactis Strain Rogers	1.5	3.8*	0.17
S. lactis X-13	0.64	0.77	0.14
None—control	0.93	5.1**	0.09

N.D. Not determined. Mixed fermentation compared to clostridium fermentation alone.
* Significant difference ($0.05 > P > 0.01$).
** Highly significant difference ($P < 0.01$). Separate fermentations compared to the control. Mixed fermentation compared to the sum of the separate butyric and propionic fermentations of the same milk.
[a] The lactic fermented milks were autoclaved prior to inoculation with the gas-formers.

to the butyric fermentation, gas production in the mixed fermentation was stimulated to a relatively lesser degree than in unfermented milk or milk fermented by less favorable lactics. Increase in rate of gas production in the mixed over the separate fermentations was more than additive in all lactic-fermented milks, except for the butyric-inhibiting *S. lactis* X-13 milk.

Effect of extent of lactic fermentation on gas production. The various lactics used in the preceding experiments fermented milk to widely differing developed titratable acidities, with the more acidogenic lactics tending to be more stimulatory to subsequent gaseous fermentations. A flask culture of *L. helveticus* in skimmilk was sampled periodically as the lactic acid fermentation proceeded. Titrations of developed acidity and direct microscopic counts were made on each sample, which was then chilled in ice water and stored at 4° C. until the parent fermentation was complete. At that time, sufficient lactic acid was added to all samples to bring their titratable acidities up to the maximum 2.8% achieved at the end of the fermentation. All samples were adjusted to pH 6.0 with $Ca(OH)_2$ and autoclaved 15 min. at 120° C. Gas evolution following inoculation with *C. tyrobutyricum* was fallowed with the gasometer. Carbon dioxide evolution by 10^9 cells of *P. shermanii* suspended in 3.2 ml. of each sample was followed manometrically with a Warburg apparatus.

The extent of the lactic fermentation had a pronounced effect on the subsequent gas-producing propionic and butyric fermentation (Table 3). Rates of gas production by both *C. tyrobutyricum* and *P. shermanii* were progressively increased as the developed titratable acidity of the lactic culture was increased. A plot of maximum rate of gas production (Figure 4) by the clostridium against per cent titratable acidity developed by *L. helveticus* revealed a linear relationship. Direct microscopic counts indicated that the number of lacto-

TABLE 3

Effect of the extent of the lactic fermentation by *L. helveticus* on the rate of gas production by *C. tyrobutyricum* and *P. shermanii* at pH 6.0 and 30° C.[a]

Titratable acidity developed by *L. helveticus*	Rate of gas production	
	C. tyrobutyricum	*P. shermanii*
(%)	(ml/10 hr.[b])	(μl/hr[c])
None—control	0.80	11
0.15	1.1	20
0.55	1.9	24
1.18	2.2	27
2.81	4.7	51

[a] All samples adjusted to 2.81% titratable acidity with lactic acid prior to adjustment to pH 5.75 with $Ca(OH)_2$.
[b] Gas measured in gasometer (Figure 1).
[c] Gas measured in Warburg apparatus.

bacillus cells reached a maximum at about 1% developed titratable acidity and remained fairly constant for the duration of the fermentation. The stimulatory effect of the *L. helveticus* fermentation on gas production by *C. tyrobutyricum* was correlated with the products or effects of energy metabolism of the lactobacilli, but apparently not with the number of lactic cells present in the fermented skimmilk. Since the concentration of lactic acid was adjusted to approximately the same level throughout the experiment, the stimulatory effect was not caused by the quantity of lactic acid.

Gas production in dialysates of lactic skimmilk culture. Dialysates were prepared simultaneously under identical conditions from portions of each of the pH-adjusted lactic cultures used in the experiments shown in Figures 2 and 3. The dialysates were crystal-clear and whey-colored, and did not form a precipitate when trichloroacetic acid was added. Rate of gas production by *P. shermanii* and *C. tyrobutyricum* in the various dialysates is shown in Table 4. Rates of gas production in the culture dialysates were determined by means of a Warburg apparatus and were compared to the rate in the dialysate of the

TABLE 4

Rate of gas production by *C. tyrobutyricum* and *P. shermanii* at 30° C. and pH 6.0 in dialysates of various lactic milk cultures

	Dialysate—μl. of gas/10 min.							
	Control	H-80	Gere A	39a	Me	DK	Rogers	X-13
C. tyrobutyricum S-12	14.9	23.7	23.0*	12.0	21.7	8.1	9.8	1.9**
P. shermanii 31C	3.4	6.2**	5.7**	4.3	3.6	3.6	3.2	3.2

* Significantly different rate from in the control (0.05 level of P).
** Highly significant different rate from in the control (0.01 level of P).
Key to milk cultures from which the dialysates were prepared:

Control—Unfermented reconstituted skimmilk
H-80—*L. helveticus* H-80
Gere A—*L. bulgaricus* Gere A
39a—*L. lactis* 39a
Me—*S. thermophilus* Me
DK—*S. faecalis* DK
Rogers—*S. lactis* Strain Rogers
X-13—*S. lactis* X-13

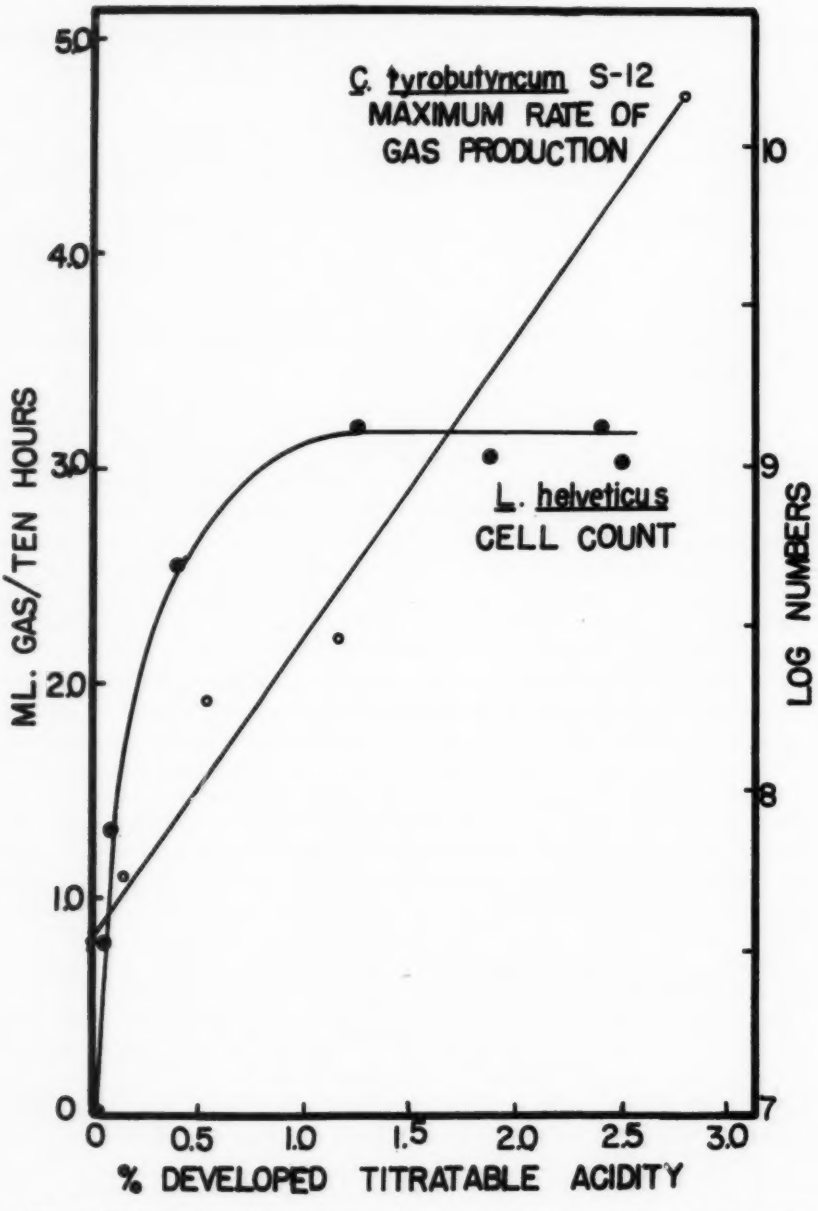

FIG. 4. Maximum rate of gas production by *C. tyrobutyricum* in relation to cell numbers and acid production of *L. helveticus*.

unfermented control milk. Gas production by *C. tyrobutyricum* was stimulated in dialysates of *L. helveticus, L. bulgaricus,* and *S. thermophilus,* and was inhibited by the dialysate of *S. lactis* X-13. No significant differences were found in the rate of gas production between the dialysates of the other cultures and the unfermented milk. These results agree closely with those for the complete culture, with the single exception of *L. lactis.* This organism was stimulatory in skimmilk culture, but its dialysate was without significant effect on the rate of the clostridial fermentation.

The rate of gas production by *P. shermanii* (Table 4) was stimulated in the dialysates of *L. helveticus* and *L. bulgaricus,* but there was no significant difference in rate between dialysates of the other cultures and of the unfermented control. This also agrees closely with the results shown with whole skimmilk cultures.

DISCUSSION

In the sequence of bacterial populations occurring in Swiss cheese, the gas-producing bacteria become active relatively late in the curing process. By that time, large numbers of bacteria of various types have already grown in the cheese and may have an effect on gas production by the propionics and butyrics. Present results indicate that the various lactic populations differ markedly in their effect on gas production by clostridia and propionics, with the normal Swiss cheese starter bacteria being particularly stimulatory for the butyric fermentation.

The complexity of the interrelationships existing between Swiss cheese bacteria was illustrated by the mixed butyric-propionic fermentation in milk that had been fermented by lactic acid bacteria. The mixed fermentation generally produced gas earlier and at a faster rate than the separate propionic and butyric fermentations; however, the basic stimulatory or inhibitory effect of the initial lactic fermentation remained.

The identity of the stimulatory and inhibitory substances in the lactic milk cultures is unknown, although it is established that they are heat- and acid-stable and of small enough molecular weight to diffuse through a dialysis membrane. The dialysate would provide a good starting material for future attempts to identify the active substances, since it is free of the colloidal and particulate matter present in the lactic milk cultures.

The origin of the stimulatory material in the milk fermented by the starter bacteria is of interest. It is possible to postulate two sources for the stimulant. The material may be a metabolic product or it might be associated with the release of lactic cell contents upon autolysis. The cell contents include enzymes which may modify the milk before heating, so as to make it more stimulatory for the gas-formers. In the present work it was shown that the stimulatory effect continued to increase in a linear manner with increase in acid production for a considerable period after the number of lactic cells had stopped increasing (Figure 4), but autolysis was not extensive. Thus, it seems unlikely that the release of cell contents was the main source of stimulant but, rather, that the stimulatory material was related to the metabolic products of the lactic organism.

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MICROESTIMATION OF IODINE IN MILK

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SUMMARY

An alkaline-ashing method is described for estimating the iodine content in milk. It can be reliably applied to determine the iodine content in 0.2-3.0 ml. of milk. Various amounts of iodide and thyroxine, added to milk samples, were successfully recovered to 102.3 and 94.0%, respectively. The standard deviation of the method, calculated from repeated processing of the same samples on different days, averaged ± 1.5 μg. (± 3.81%) of total iodine per 1,000 ml. of milk. The analytical results were confirmed by the biological control.

Several procedures have been proposed for estimating iodine in biological materials and these may conveniently be grouped into two main categories: Mineral-distillation methods (2, 6, 8), whereby the organic substance is destroyed in sulfuric acid and the iodine released is oxidized with potassium permanganate or chromic anhydride. The iodate resulting from the reaction is reduced with oxalic or phosphorous acids and the liberated iodine is distilled into an alkaline closed system, part of which is used for the colorimetric determination. In the alkaline-ashing methods (1, 3, 12) the organic substance is destroyed by high temperatures and the liberated iodine is bound to the alkali. The residue is dissolved in acids and the iodine content is colorimetrically determined from the supernatant.

The procedure most commonly used is that of Sandell-Kolthoff's reaction (7) in which the iodine catalyzes the oxidation-reduction reaction

$$2\,Ce^{4+} + As^{3+} \xrightarrow{J_2} 2\,Ce^{3+} + As^{5+}.$$

Both procedures, partly modified, have been routinely used for several years in this laboratory (4). However, in spite of several adaptations, they have proved inadequate for the exact assay of the iodine content in milk—a fact due probably to the high content of various substances such as fats and proteins. Of the two, the alkaline-ashing method gave better results and appeared more suitable, not only on account of its greater precision but also because it required less time and chemicals. In spite of initial failures to obtain satisfactory results, attempts were made to develop a method that would suit our purpose. Therefore, the alkaline-ashing method was examined thoroughly and the optimum conditions for the catalytic action of iodine in the Sandell-Kolthoff's reaction were determined (10). In addition, the effect of various ions on this particular reaction were studied (11), as it was suspected that errors in the results might be due to the appearance of different ions in milk samples.

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To verify our method, various amounts of milk were used for the analysis; further, iodide and thyroxine were added to milk samples, and parallel series of the samples were processed on different days, thus making it possible to determine the margin of error. In addition, the ratio of protein iodine and of whole iodine in milk also was investigated. A certain measure of biological verification was assured by processing samples of protein-bound iodine of human subjects and comparing the iodine content of milk from regions of endemic and enzootic goiter.

METHODS AND PROCEDURES

Reagents:

1. Sodium carbonate—2 *N*.

2. Acid mixture—120.5 ml. of concentrated sulfuric acid are added to 500 ml. of redistilled water. 58.5 g. of sodium chloride dissolved in 200 ml. of water, and together with 4.9455 g. of arsenic trioxide dissolved in 20 ml. of 7% potassium hydroxide are added into diluted sulfuric acid and the volume is brought to 1,000 ml. with water.

3. Ceric–ammonium sulphate—0.005 *M*. 3.1625 g. of ceric salt (Ce^{4+}) are suspended in 500 ml. of water and 80.5 ml. of concentrated sulfuric acid are added under constant stirring. When dissolved the whole is filtered through a glass filter and made up to 1,000 ml. with water.

4. Brucin acetate—1%. 5.0 g. of the base are suspended in 200 ml. of water; 6.0 ml. of ice-cold acetic acid are added and when dissolved the contents are filtered. The volume is brought to 500 ml. with water.

5. Zinc sulphate—10%.

6. Basic iodine solution—10 mg. %. 130.8 mg. of potassium iodide (or 118.1 mg. of sodium iodide) are dissolved in 1,000 ml. of water (1 ml. = 100 μg. of iodine).

a) Intermediary iodine solution—2.5 ml. of the basic iodine solution is made up to 1,000 ml. of volume with water (1 ml. = 0.25 μg. of iodine).

b) Working iodine solution—calibration curve. 33.0 ml. of 2 *N* sodium carbonate are added into 1.0, 2.0, 4.0, 6.0, and 8.0 ml. of the intermediary solution and the contents are made up to 100 ml. with water. A blank is prepared by making up the volume of 33.0 ml. of 2 *N* sodium carbonate to 100 ml. with water. In 2.0 ml. of these solutions there are the following amounts of iodine: 0.000, 0.005, 0.010, 0.020, 0.030, and 0.040 μg. The blank of calibration curve is also used for diluting samples containing more than 120.0 μg. of iodine per 1,000 ml. of milk.

Working procedure:

A mixture of 1.0 ml. of milk, 1.0 ml. of 10% zinc sulphate, and 2.0 ml. of 2 *N* sodium carbonate are left to dry on a boiling bath or overnight in a desiccator. The test tubes are heated in a muffle-furnace on a china clay dish at 600 $\pm$ 10° C. for 150 min. When cooled, the residuum is suspended in 6.0 ml. of water with the help of an electric motor. The samples are centrifuged for

10 min. at 2,500 r.p.m. 2.0 ml. of pure supernatant are taken, 2.0 ml. of the acid mixture are added and, after a thorough mixing, the stands with the samples are placed in an ice-bath (+ 4° C.). After 10 min., 2.0 ml. of an equally cooled cerium solution are added. When the cerium solution has been added to all the samples, the contents of the test tubes are thoroughly mixed and they are all placed at the same time into a warm bath (40 ± 0.1° C.) for 20 min. Then they are again transferred into the ice-bath (+4° C.) and here after 10 min. 0.5 ml. of 1% brucin solution are added. The samples are mixed and placed for 15 min. in a thermostat heated to 100° C. A stable color develops which may be read on a spectrophotometer at 430 mμ up to 48 hr.

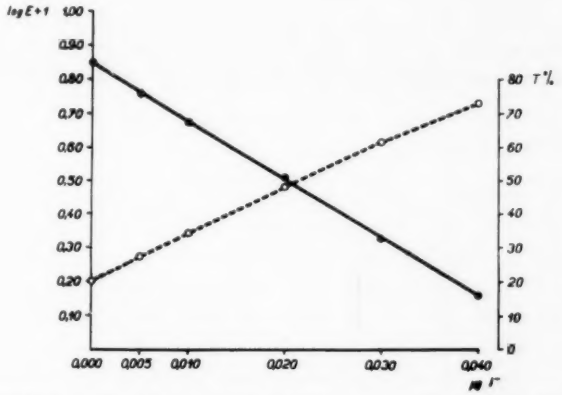

FIG. 1. Standard curve for iodine determination. Relation between log extinction and iodide concentration (———) and percentage of transmission and iodide concentration (- - - -).

A calibration curve is constructed with every colorimetric series (30-50 milk samples). This curve follows a linear course when the log of extinction is plotted on a millimeter paper against iodine concentration (Figure 1). The corresponding iodine values are read off this curve and calculated for 1,000 ml. of milk.

VERIFICATION OF THE METHOD FOR IODINE ESTIMATION IN MILK

The use of varying amounts of milk. Amounts of 0.2, 1.0, 2.0, and 3.0 ml. of full-cream milk were taken and processed as described above. The sample containing 0.2 ml. of milk was ashed in a muffle-furnace for 60 min. and the residue dissolved in 3.0 ml. of water; 2.0 ml. of the pure supernatant were used for the colorimetric estimation of iodine.

Recovery of added iodide. Amounts of 0.015 and 0.030 μg. of iodide were added to the 1.0-ml. milk samples containing 45.1, 46.2, 54.3, and 40.2 μg. of iodine in 1,000 ml. To the 2.0-ml. milk sample 0.030 μg. of iodide was added. The procedure as described was carried out.

Recovery of added thyroxine. To the sample of 1.0 ml. of milk, containing 32.5 and 54.7 μg. of iodine per 1,000 ml., were added 0.0162, 0.0324, 0.0486, and 0.0648 μg. of iodine in the form of iodinated amino acid–thyroxine.

Effect of the ashing and colorimetric procedure on the standard deviation (S.D.) of the method. A series of 37 (1.0 ml.) milk samples were processed as stated above. In addition, they were ashed, nine or ten at a time, in a muffle-furnace. The colorimetric procedure was likewise carried out on nine or ten samples at a time and the results were read off four calibration curves.

Ratio of protein and total iodine in milk. Values for the protein iodine were obtained as follows: 10.0 ml. of redistilled water were added to 1.0 ml. of milk and then were also added 1.0 ml. of 10% zinc sulphate and 1.0 ml. of 2% sodium hydroxide. The mixture was thoroughly mixed after the addition of every ingredient and on completion of this operation it was left to stand for 60 min. at room temperature. The samples were then centrifuged for 10 min. at 2,500 r.p.m., the supernatant decanted, and 2.0 ml. of 2 *N* sodium carbonate added,

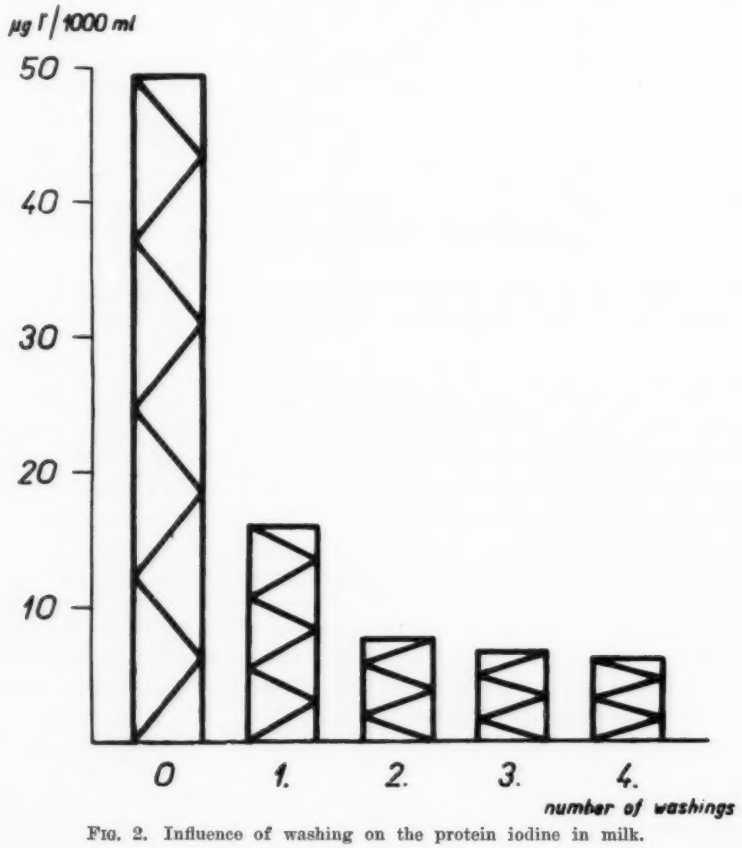

FIG. 2. Influence of washing on the protein iodine in milk.

or a further 10.0 ml. of redistilled water (the latter was added in accordance with the number of washings of the milk proteins to eliminate inorganic iodide). In the present experiments, the samples were washed 1, 2, 3, and 4 times. The remaining steps are identical with those used for total iodine determination in milk.

Biological control of the method. Samples of serum protein-bound iodine from subjects with various functional states of the thyroid were processed in the same way. In a further experiment, the iodine content in cow's milk, from different regions of Slovakia, with varying degrees of endemic and enzootic goiter, was determined.

RESULTS

Table 1 summarizes the results obtained by using different quantities of milk for the iodine estimation. It will be noted that both microquantities, represented by fractions of a milliliter, and macroquantities consisting of several milliliters of milk, may be processed for iodine determination. The recovery of iodide, added to the milk samples, amounted on an average to 102.3%. On the other hand, if various amounts of thyroxine had been added, the average recovery was around 94.0%. Results are shown in Tables 2 and 3.

Samples of the same milk supply, which had been ashed or processed colorimetrically in batches, showed an average range of S.D. of $\pm$ 3.82%. The average iodine value in the first batch of samples was 39.5, with a S.D. of $\pm$ 1.8; the second batch average, 40.1 $\pm$ 0.9; the third, 38.6 $\pm$ 1.4; and the fourth, 39.2 $\pm$ 1.9 μg. of iodine per 1,000 ml. of milk. The average value for 37 milk samples, processed on different batches, was 39.3 $\pm$ 1.5 μg. of iodine per 1,000 ml. of milk. Detailed results are shown in Table 4.

Data obtained in estimating protein iodine in milk show that inorganic iodide or iodinated compounds weakly bound to milk proteins may be eliminated with two washings with 10 ml. of redistilled water. From the results (Table 5), it appears that protein iodine in milk is about 15% of all the iodine in cow's milk.

TABLE 1

Various amounts of milk used in the analysis

Sample	Milliliter of milk	μg. I/1,000 ml.	In per cent
M1	1.0	38.1	100.0
M1	2.0	36.0	94.5
M1	3.0	35.5	93.2
M2	1.0	45.1	100.0
M2	2.0	45.4	100.7
M2	3.0	42.1	93.3
M3	1.0	46.2	100.0
M3	2.0	46.2	100.0
M3	3.0	46.5	100.7
M4	1.0	170.0	100.0
M4	0.2	157.5	92.6
M5	1.0	20.6	100.0
M5	0.2	18.7	90.8
M6	1.0	62.9	100.0
M6	0.2	64.6	102.7

TABLE 2

Recovery of inorganic iodide added to the milk samples

Sample	Milliliter of milk	μg. I added	μg. I/1,000 ml. Found	μg. I/1,000 ml. Theoretically	In per cent
1	1.0	.....	45.1	.....	.....
1	1.0	0.015	63.0	60.1	105.0
1	1.0	0.030	81.0	75.1	107.9
1	2.0	0.030	62.7	60.1	104.3
2	1.0	.....	54.3	.....	.....
2	1.0	0.015	67.5	69.3	97.4
2	1.0	0.030	85.1	84.3	101.0
3	1.0	.....	46.2	.....	.....
3	1.0	0.015	62.5	61.2	102.2
3	1.0	0.030	77.7	76.2	102.0
3	2.0	0.030	63.1	61.2	103.1
4	1.0	.....	40.2	.....	.....
4	1.0	0.015	54.7	55.2	99.1
4	1.0	0.030	72.3	70.2	103.0

TABLE 3

Recovery of thyroxine added to the milk samples

Sample	Milliliter of milk	μg. I added [a]	μg. I/1,000 ml. Found	μg. I/1,000 ml. Theoretically	In per cent
1	1.0	.....	32.5	.....	.....
1	1.0	0.0162	46.0	48.7	94.5
1	1.0	0.0324	58.7	64.9	90.4
1	1.0	0.0486	68.8	81.1	84.8
2	1.0	.....	54.7	.....	.....
2	1.0	0.0162	66.7	70.9	94.0
2	1.0	0.0324	85.5	87.1	98.1
2	1.0	0.0486	96.7	103.3	93.6
2	1.0	0.0648	108.5	119.5	90.7

[a] In the form of thyroxine.

TABLE 4

Iodine estimation in parallel milk samples ashed and processed by colorimetry on different days

Batch 1st	2nd	3rd	4th
41.0[a]	39.0	37.0	39.0
41.0	40.5	37.0	40.0
37.0	40.5	39.0	40.0
40.5	40.5	41.0	40.0
37.5	39.0	39.0	36.0
40.5	39.0	39.0	41.0
40.5	41.0	40.0	36.0
37.0	41.0	37.5	41.0
41.0	41.0	40.0	40.0
.....	.....	37.0	.....
$\bar{x}$ 39.5	40.1	38.6	39.2
S.D. ± 1.8	± 0.9	± 1.4	± 1.9
$\bar{x}$ ± S.D.	39.3 ± 1.5		

[a] The values are given in μg. of I/1,000 ml.

In estimating protein-bound iodine levels in healthy subjects, we obtained values ranging from 3.0 to 7.0 μg. % of PBI, which corresponds to the values generally stated in the literature. These results have been published in more detail elsewhere (9). Likewise, the results of a close correlation found to exist between the iodine content in milk and the incidence of endemic and enzootic goiter were published in another paper (5).

The analytical results and the biological control of the method, carried out as described, show that we have succeeded in working out a precise and reliable procedure for routine analyses of iodine content in milk.

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WHEY PROTEINS OF DRYING-OFF SECRETIONS, MASTITIC MILK, AND COLOSTRUM SEPARATED BY ION-EXCHANGE CELLULOSE [1]

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SUMMARY

Quarter milk samples from three cows in late lactation, dried off by intermittent milking, were analyzed for chlorides, pH, breed cell count, California Mastitis Test (CMT) score, and whey proteins. As milk production dropped, a rise in values for all constituents was observed. Increased whey protein was principally immune globulins resolved on DEAE-cellulose as four separate but probably nonhomogeneous components. The udder-formed proteins were secreted at a constant rate during the drying-off phase.

Similar changes were observed in a case of induced mastitis. At 8 hr. a marked rise in blood serum albumin was found. The quarter then became nonfunctional. At 72 hr. the whey protein pattern was similar to the latter drying-off secretions. In both cases, the principal fraction was an immune-globulin that was not adsorbed on to the column.

Though the relative concentrations of individual proteins of colostral whey were similar in magnitude to mastitic milk and latter drying-off secretions, the nonadsorbed immune globulin was markedly decreased and the bulk of the immune globulins appeared as a peak eluting with 0.05-0.075 *M* NaCl. A part of the difference was a colored component present in mastitic milk and drying-off secretions, but absent or in low concentration in colostrum.

Euglobulin and pseudoglobulin prepared by two methods produced higher euglobulin/pseudoglobulin ratios from drying-off secretions than from colostrum. The amount of euglobulin and pseudoglobulin found and the elution pattern from DEAE-cellulose varied with the method and source. Thus, no peak could be described as principally a euglobulin or pseudoglobulin.

It has been observed in this laboratory that a proportionately higher number of cows in late lactation produce milks giving California Mastitis Test (CMT) positive reactions, compared with milks from normal cows in early- or mid-lactation. Secretions of dry cows obtained for routine bacteriologic examination also usually are positive to the CMT.

Of the many changes occurring in milk from mastitic udders, those in the whey proteins are well known (6, 10, 13), in that the amounts of the blood-formed proteins increase with the severity of the disease. Similar changes occur in colostrum and drying-off secretions of normal cows (8, 9).

Many proteins have been shown to undergo complex formation, denaturation, and aggregation in the presence of synthetic detergents (11), the active component of the CMT reagent (14). These reactions suggested a possible relationship between the altered composition of whey proteins of mastitic milk, drying-off secretions, and the CMT reaction.

Ion-exchange cellulose was chosen for separation of whey proteins, since

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individual proteins can be resolved into solutions that are relatively homogenous. It was found early, however, that though protein is involved in the CMT reaction, separated whey proteins per se do not produce reactions typical of CMT-positive milks. The trends observed, however, warranted further study.

Data are presented on the relative concentration of individual whey proteins during various stages of the early drying-off period and the effect of previous udder infection on the drying-off secretions. In addition, observations comparing whey proteins from drying-off secretions, mastitic milk, and colostrum are presented, using DEAE ion-exchange cellulose.

METHODS AND PROCEDURES

The cows represented here are a part of a herd maintained for mastitis research in which the effects of various mastitis-producing agents injected into the udder are being studied. These investigations will be reported elsewhere. In all cases, the cows were high-producing, pure-bred Holsteins. For the drying-off studies, three cows in the latter stages of lactation were selected. With the exception of the left front quarters of Cows 2414 and 2365, all quarters had been subjected to induced mastitis in the second month of lactation. Milk production was depressed for approximately 1 wk. from Cow 2414, but production was not effected in Cows 2365 and 2333 and in no case did clinical symptoms persist beyond a week in any quarter. For the studies reported, the cows were considered normal since milk production was normal; they were clinically sound and the udders were bacteria-free throughout the study. The two quarters of Cows 2414 and 2365 mentioned were healthy throughout the lactation.

On September 26, the cows were changed from regular twice-a-day milking to once a day till September 30. They were milked once every other day till October 7. All cows were sampled (250 ml.) on October 10 and Cow 2414 was sampled on October 13. Since each cow was producing several pounds on October 7, the glands were probably not completely dry on October 10. Cow 2365 was in the 247th, 2414 in the 227th, and 2333 in the 248th day of lactation, respectively. The analyses reported represent milks from the first 150-200 ml. of each milking from each quarter. For simplicity, when viewed from the right, Quarters A and B represent the right front and rear, C and D the left front and rear, respectively.

Cow 44 was in the third month of the second lactation. The experimental infection was produced by inoculation of all quarters with a 1-ml. saline suspension of a dilution of an 18-hr. broth culture of *Aerobacter aerogenes*, which was isolated in August, 1950, from an acute case of mastitis occurring in a commercial dairy herd. Mastitis of varying degrees of severity was produced in all quarters. Details from only the A quarter will be presented.

Whey proteins were separated on DEAE-cellulose by the method of Yaguchi *et al.* (18), using either N_2 or hydrostatic pressure for step-wise elution. Optical densities of the 20-ml. eluate fractions were read at 280 mμ on the Beckman DU Spectrophotometer. The elution curves were quantitated by plotting the O.D. readings on good quality graph paper and weighing the amount of paper representing individual components.

Wheys representing 50 ml. of milk were added to the column in the case of the drying-off and mastitic milks. Smaller volumes of colostrum were used in view of the higher whey protein content.

The elution position of blood serum albumin was verified by the addition of authentic bovine blood serum albumin to the column separately, to one of the wheys, and by comparison with paper electrophoresis data.

Total whey protein was determined by the biuret reaction. Fifty milliliters of skimmilk was brought to pH 4.6 with *N* HCl–acetic acid (1:1 by volume). After standing several hours, the whey was separated by filtration and the casein curd was washed with several volumes of water. Upon neutralization to pH 6.8 with *N* NaOH, the whey was diluted to 250 ml., and the protein from 5-ml. aliquots of this dilution was precipitated with 15% trichloroacetic acid. The precipitates were collected by centrifugation, dissolved to 10 ml. with *N* NaOH, and protein was determined from 5 ml. of the final solution after diluting with an equal volume of biuret reagent. The color was read at 540 mμ. Protein concentrations were obtained by comparison with a standard curve prepared with standardized bovine serum albumin.

The paper electrophoresis method was that of Leece and Legates (10).

EXPERIMENTAL RESULTS

Separation and identification of whey proteins. Yaguchi *et al.* (18), using step-wise elution, found that nine peaks could be resolved from whey with NaCl concentrations from 0.0 to 0.20 *M* in 0.02 *M* phosphate buffer. Preliminary trials in the present investigation indicated that even more peaks could be resolved, depending on the buffer volumes used. If but 0.15 *M* NaCl was used to elute β-lactoglobulin (the final peak) from wheys containing but one β-lactoglobulin species, a marked tailing-off occurred. Whereas, if 200-ml. volumes of 0.15, 0.175, and 0.20 *M* NaCl were used to elute the β-lactoglobulin from whey from the same cow, three distinct peaks were produced. The ascending limb of each peak corresponded to the point of emergence of the next strongest buffer. When purified blood serum albumin was chromatographed alone, if but one buffer was used (0.125 *M* NaCl in 0.02 *M* phosphate), a pointed Gaussian curve was produced. However, if a stronger buffer was introduced before the albumin was completely eluted, an additional spike, corresponding to the emergence of the stronger buffer, was produced on the descending limb of the curve.

With the range of protein concentrations encountered (*ca.* 0.75-1.5 g.) symmetrical peaks were produced with 300-ml. volumes of buffer. However, as the relative concentration of α-lactalbumin declined, this volume usually produced flattened plateaus on the ascending limb of the α-lactalbumin peak (Peak 5, Figures 1D and E).

Four distinct peaks (1-4) could be eluted from all except normal wheys with buffers containing 0.0 to 0.1 *M* NaCl. Peaks 1 and 2 (eluted with 0.0 and 0.05 *M* NaCl in phosphate buffer, respectively) were always present in normal wheys. That Peaks 3 and 4 were real was judged by the fact that they were separated from adjacent peaks on many occasions by the same buffer. (In Figure 6, Peak 2

eluted with Peak 3 with 0.075 *M* NaCl, and in Figures 1B, 1C, 1D, and 4C, Peak 4 and Peak 5 eluted with 0.10 *M* NaCl.) It was never possible to completely resolve Peaks 4 and 5.

Presumptive evidence indicated Peaks 1-4 were immune globulins. When the pervaporated fractions were subjected to paper electrophoresis, each fraction migrated within the γ-globulin region of blood serum and the immune globulin region of whey. Values for total immune globulins from whey separated on DEAE agreed well with those separated by paper electrophoresis and quantitated on the Analytrol, though it was recognized that different entities were being measured in both cases. Differences in electrophoretic mobility between the four fractions could not be detected.

Since the criteria of separating the immune globulins of milk into eu- and pseudoglobulin have been adopted (1), attempts were made to identify the peaks as one or the other. It was found that varying results were obtained, depending on the method of preparation. The following ratios of euglobulin/pseudoglobulin were found: Method of McDougall (12)—colostrum 1:0.012, drying-off milk, 1:1.5, and mastitic milk, 1:3; method of Smith (15)—colostrum, 1:1.4 and drying-off milk, 1:4.5. Regardless of the method of preparation, euglobulin and pseudoglobulin produced Peaks 1-4 when separated on DEAE. However, with McDougall's method, Peak 2 predominated from both colostral eu- and pseudoglobulin (*ca.* 54% of the total of Peaks 1-4) in each case and, with Smith's method, Peak 2 represented 59% of the total of Peaks 1-4 from euglobulin and 44% from pseudoglobulin of colostrum. Peak 1 predominated from drying-off euglobulin by Smith's method (53% of the total of Peaks 1-4) and Peak 2 was the largest fraction (48% of the total of Peaks 1-4) from pseudoglobulin.

It was observed on several occasions that two sharp peaks (usually involving but one-two tubes) were found in the breakthrough fraction (eluted by the starting buffer). It was not possible to determine if these were due to column behavior. Both pseudo- and euglobulin fractions (Smith's method) from colostrum produced two peaks in this region and but one symmetrical peak was produced from drying-off milk.

In all cases, when absorbancies of tubes from Peak 1 from mastitic milk and drying-off secretions were above 0.5, the solutions were colored a faint pinkish-brown and the lyophilized product was decidedly pink. Similar tubes from colostrum contained less to no color, even at much higher concentrations of protein. Colostral euglobulins prepared by McDougall's method were not colored in the Peak 1 region, whereas similar tubes from pseudoglobulin were, yet the concentration of the latter was 5.6 times less than that of the former. Neither fraction prepared from Cow 2414 colostrum by Smith's method showed a colored component in the Peak 1 region. It was present in euglobulin of drying-off milk from the same cow prepared by this method, however. Experiments are in progress in an effort to characterize this colored component. Evidence to date suggests it is not hemoglobin.

Difficulty was experienced in effectively separating blood serum albumin from β-lactoglobulin if both Lactoglobulins A and B were present. It was found that 0.125 *M* NaCl would elute blood serum albumin; however, with this concen-

tration, the serum albumin peak (Peak 6) would tend to tail off and merge with the β-lactoglobulin peaks. By using 0.163 *M* NaCl, the serum albumin peak was eluted sharply. The same situation obtained for β-lactoglobulin. If no serum albumin was present, the lactoglobulin peaks would begin to elute with 0.15 *M* NaCl, but extensive tailing-off resulted. Accordingly, for the separations reported here, NaCl concentrations of 0.213 to 0.225 *M* were used to elute the lactoglobulins, since one sharp peak resulted, making extrapolation easier. The method is effective in separating both lactoglobulins (see Figure 6, Peaks 7 and 8).

In some cases, where blood serum albumin was in low concentration and the cow produced both species of β-lactoglobulins, the serum albumin peak would be masked completely. (The presence of serum albumin was confirmed in several of these samples by electrophoresis.) As the concentration of blood serum albumin rose, two peaks in the serum albumin region and a shoulder on the ascending limb of the β-lactoglobulin peak invariably occurred (Figure 4C). In these cases, quantitative comparison with samples separated by paper electrophoresis indicated the values were similar if the first peak was included with blood serum albumin, the second with β-lactoglobulin. As the concentration of serum albumin increased still further, and the lactoglobulin concentration fell, the albumin peak sharpened considerably and the shoulder on the ascending limb of the lactoglobulin disappeared.

Gradient elution with one or two mixing chambers and several different volumes of buffer was tried. Normal wheys were separated effectively, but Peaks 1, 2, and 6 of abnormal wheys were broadened to the point where extrapolation was difficult.

Drying-off secretions—chlorides, pH, cell-count, and *CMT score.*

The values for these constituents plus total milk production during the drying-off period are shown in Table 1. Values for chlorides and pH for C quarters of Cows 2414 and 2365 are listed separately for comparison, since these quarters were mastitis-free during the lactation under study.

The data show a progressive, though variable increase in chlorides, pH, cell-count, and CMT score as the udders decline in production. All values for the two C quarters tended to remain lower, though the differences in chlorides and pH are probably not statistically significant. The lowered cell count and CMT score in these quarters is quite marked, however. The observation that mastitis-free quarters tend to remain CMT-negative longer in the dry period than quarters which suffered mastitis attacks, has been observed repeatedly in this laboratory. The high cell counts in the latter stages in Cow 2333 are probably due to the concentration effect of the more severe drop in milk production.

Drying-off secretions—whey proteins. As milk production dropped, a sharp rise in the relative per cent of immune globulins and a steady increase in blood serum albumin was found (Table 2). Increased concentration of these proteins was accompanied by a corresponding decrease in the udder-formed proteins (principally β-lactoglobulins). A threefold increase in relative concentration of immune globulins and a fourfold increase in blood serum albumin are evident.

TABLE 1

Chloride content, pH, cell count, CMT score, and total milk production during the drying-off period

	9/26	9/28	9/30	10/3	10/5	10/7	10/10	10/13
				% chlorine				
Quarter								
All quarters	0.15	0.16	0.19	0.25	0.25	0.26	0.33	0.39
2414 C	0.14	0.16	0.16	0.24	0.20	0.22	0.30	0.35
2365 C	0.18	0.15	0.18	0.18	0.20	0.23	0.29	
Range	0.14–0.19	0.15–0.19	0.18–0.31	0.18–0.31	0.17–0.30	0.22–0.30	0.29–0.38	
				pH				
All quarters	6.66	6.64	6.65	6.84	6.84	6.96	7.26	7.43
2414 C	6.68	6.58	6.55	6.64	6.72	6.72	6.95	7.60
2365 C	6.60	6.62	6.65	6.70	6.82	6.71	6.90	

Breed Cell Count × 10^4 (Column 1) and CMT Score (Column 2)

	pH 6.55–6.77		6.58–6.73		6.59–6.77		6.64–6.89		6.72–6.89		6.71–7.17		6.90–7.47		7.26–7.60	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
2414 A	0	Neg.	8	Neg.	6	Neg.	12	Neg.	16	Neg.	8	T	16	T	1,040[a]	3[a]
B	2	Neg.	8	Neg.	2	Neg.	20	T	50	1	54	1	30	1	1,240[a]	3[a]
C	2	Neg.	2	Neg.	0	Neg.	0	Neg.	2	Neg.	0	Neg.	8	Neg.	3,000[a]	3[a]
D	6	Neg.	26	T	38	T	26	1	216	2	42	T	30	1	1,200[a]	3[a]
2365 A	8	T	2	Neg.	16	T	16	T	48	1	230	2	12	1		
B	10	Neg.	24	1	58	1	6	Neg.	72	1	166	2	66	2		
C	4	Neg.	0	Neg.	4	Neg.	0	Neg.	12	Neg.	2	Neg.	8	T		
D	10	Neg.	30	T	30	T	44	1	62	1	40	1	56	2		
2333 A	2	Neg.	6	Neg.	28	Neg.	16	Neg.	280	2	660	3	394	3		
B	4	Neg.	10	Neg.	12	Neg.	16	Neg.	144	1	300	2	320	2		
C	6	Neg.	4	Neg.	8	Neg.	6	Neg.	10	Neg.	192	2	286	2		
D	4	Neg.	4	Neg.	18	Neg.	6	Neg.	16	Neg.	140	1	108	2		

Milk Production *(lb.)*

2414	25.0	15.5	12.0	16.5	12.0	12.5
2365	20.5	12.0	8.5	11.5	9.0	11.0
2333	21.0	12.0	10.0	7.5	5.0	5.5

[a] Fore milk (first 30 ml.).

TABLE 2

Relative per cent of individual whey proteins during the drying-off period

Day[a]	Cow 2333 A	Cow 2333 B	Cow 2333 C	Cow 2333 D	Cow 2414 B	Cow 2414 C	Cow 2414 D	Cow 2365 A	Cow 2365 C	Cow 2365 D	Average
					Immune globulins						
1	27.8	21.0	17.3	17.3	18.7	20.3	17.1	15.2	16.1	17.0	18.6 ± 1.45
4	.....	.....	.....	.....	29.0	19.2	.....	41.3	.....	37.6	34.9 ± 5.21
7	39.5	33.7	47.2	31.6	44.0	.....	30.6	.....	33.3	33.7	39.9 ± 2.14
9	43.0	54.0	.....	39.9	.....	40.2	.....	47.7	.....	.....	44.9 ± 2.44
11	.....	.....	52.6	49.0	48.7	42.1	51.2	43.8	41.8	45.6	47.0 ± 1.46
14	57.0	61.7	54.2	57.0	57.1	50.1	54.8	49.3	46.7	47.3	53.5 ± 1.55
17	.....	.....	.....	.....	.....	57.7	63.8	.....	.....	.....	60.8
					α-Lactalbumin						
1	21.3	25.5	33.0	33.0	30.9	27.9	34.0	24.5	23.6	22.7	26.9 ± 1.29
4	.....	.....	.....	.....	22.6	26.2	.....	23.2	.....	18.1	22.5 ± 2.11
7	15.4	13.7	22.1	17.8	24.5	.....	15.8	.....	22.2	19.2	18.8 ± 1.46
9	11.8	11.4	.....	14.0	.....	21.6	.....	15.8	.....	.....	14.9 ± 1.85
11	.....	.....	9.8	13.7	17.9	16.8	13.9	17.6	22.5	17.4	16.2 ± 1.33
14	10.4	9.0	10.0	11.0	12.9	15.7	11.7	16.6	14.5	17.7	12.9 ± 0.95
17	.....	.....	.....	.....	.....	9.6	8.8	.....	.....	.....	9.2
					Blood serum albumin						
1	2.3	6.6	6.3	6.3	0.0	0.0	0.0	6.4	0.0	5.2	3.1 ± 0.98
4	.....	.....	.....	.....	0.0	5.6	.....	0.0	.....	6.0	3.9 ± 1.77
7	13.7	6.9	0.0	9.5	4.4	.....	6.4	.....	1.6	10.6	6.6 ± 1.69
9	14.2	10.1	.....	10.5	.....	6.2	.....	7.4	.....	.....	9.7 ± 1.40
11	.....	.....	11.0	9.3	11.2	9.1	12.2	6.5	9.4	9.0	9.6 ± 0.62
14	16.6	11.9	12.4	18.5	9.9	7.4	14.4	13.2	8.9	17.1	12.9 ± 1.08
17	.....	.....	.....	.....	.....	18.1	18.9	.....	.....	.....	18.5
					β-Lactoglobulin						
1	48.7	47.0	43.4	43.4	50.8	51.8	49.7	53.9	54.6	55.2	50.9 ± 1.40
4	.....	.....	.....	.....	48.5	48.9	.....	35.5	.....	38.3	42.8 ± 3.46
7	32.3	45.7	30.7	41.1	27.1	.....	37.2	.....	42.9	36.5	36.7 ± 2.26
9	31.0	24.5	.....	35.5	.....	32.0	.....	29.1	.....	.....	30.4 ± 1.83
11	.....	.....	26.6	27.9	22.2	31.0	22.7	32.1	26.3	28.0	26.7 ± 1.24
14	16.1	17.4	23.5	14.6	20.1	26.7	19.2	21.0	29.9	18.0	20.7 ± 1.52
17	.....	.....	.....	.....	.....	14.6	8.6	.....	.....	.....	11.6

[a] Day 1 represents September 26, the first day of the interrupted milking schedule; Day 4 represents September 30, etc.

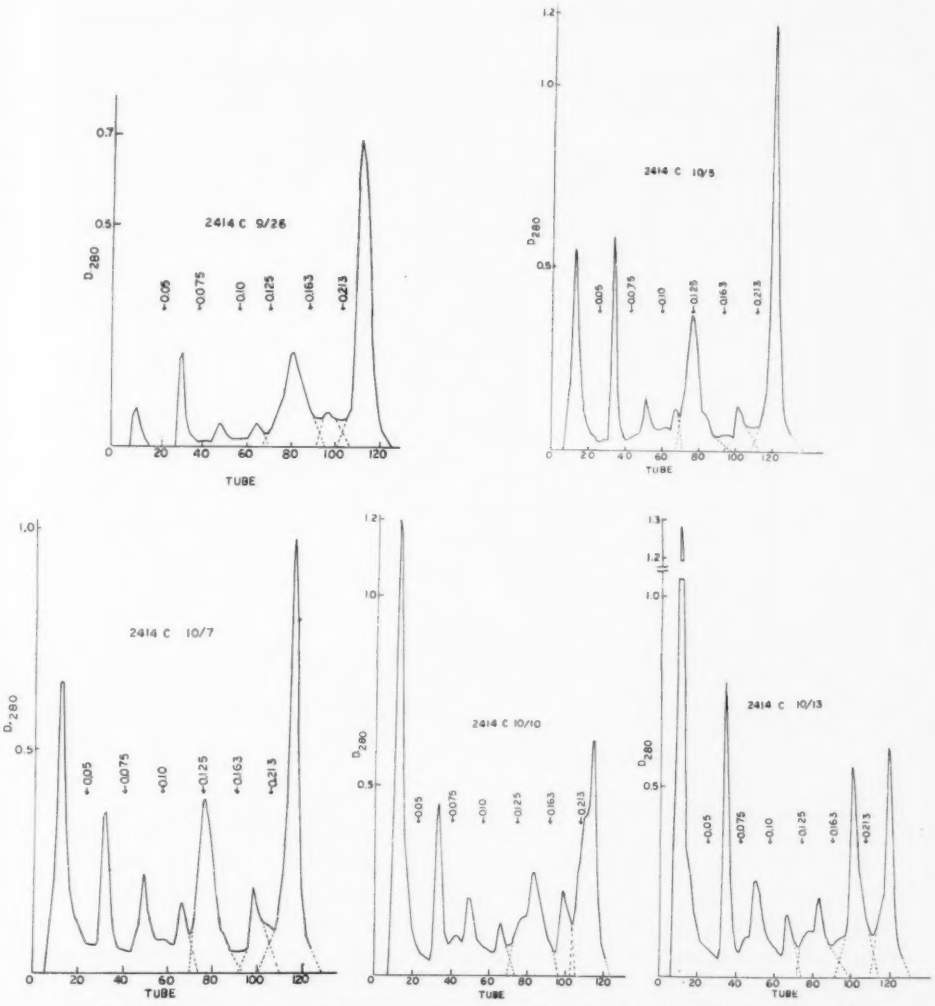

FIG. 1A. Elution diagrams of wheys representing 50 ml. of milk obtained on the dates indicated. Left to right, Peaks 1-4 = Immune globulins, Peak 5 = α-Lactalbumin, Peak 6 = Blood serum albumin, and Peak 7 = β-Lactoglobulin. Vertical figures in these and elution diagrams to follow refer to the molar concentration of NaCl in 0.02 *M* phosphate buffer added at that point.

Elution curves of one quarter (Figure 1) show that increased immune globulins were principally in Peak 1. This was true for each series of drying-off milks examined. This fraction amounted to as much as 40% of the total whey protein on October 10.

Differences in values for the C quarters of Cows 2414 and 2365 and the corre-

sponding values from quarters which had suffered mastitis attacks are probably not significant.

A least-squares plot of the average relative per cent concentration of individual proteins was constructed (Figure 2), and absolute values for individual

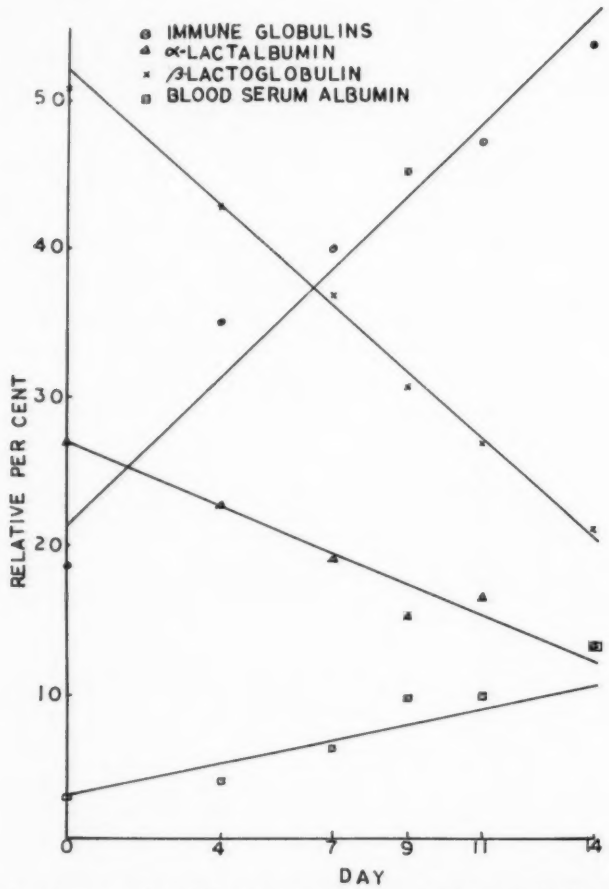

FIG. 2. Least-squares plot of the average relative per cent concentration of individual whey proteins. The days refer to the number of days after the interrupted milking schedule was initiated (September 26).

proteins for the various times were calculated and plotted (Figure 3) from the total whey values obtained from one quarter (the A quarter, Cow 2414).

Though extrapolation of the total whey protein curve to zero time indicates levels of 0.36%, values from other quarters were between 0.4-0.5% on September 26. The curves indicate that total whey protein rises as milk production falls and that increased protein is principally immune globulin(s). A small but

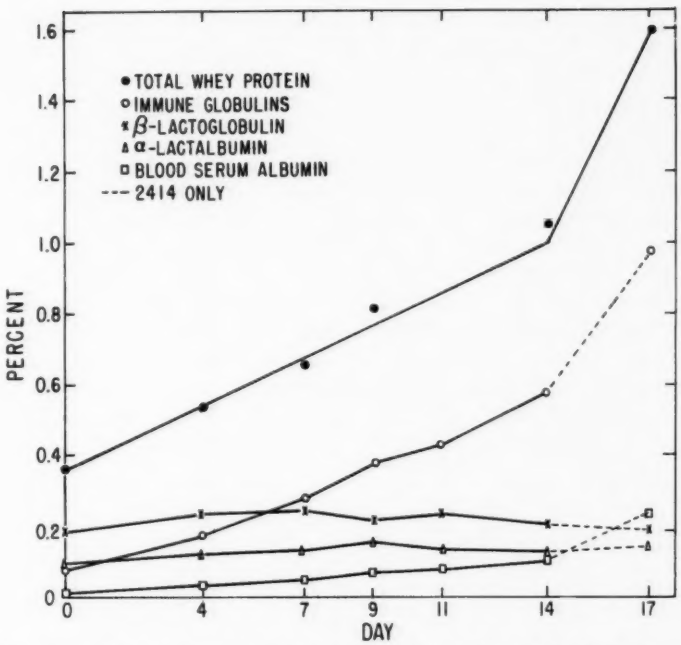

FIG. 3. Absolute amount of total whey protein and of individual whey proteins as per cent by volume of the original milk. Total whey protein was determined on secretions from one quarter (2414 A) and individual whey protein concentrations were calculated from this curve using the relative values obtained from Figure 2. The days refer to number of days after the interrupted milking schedule was initiated (September 26).

persistent rise in blood serum albumin is evident. The most abrupt change was between October 10 and 13 (Cow 2414), a period during which milk production had practically ceased. In spite of the drop in total milk produced, the udder-formed proteins appear to be secreted at a constant rate.

Colostral whey proteins. Colostrum from two cows (2414 and 2481) was chromatographed. In both cases, the colostrum was obtained on the day of parturition, before the calves had access to the dam. The curves obtained (Figures 6 and 7) show a marked difference between colostrum, late drying-off secretions, and mastitic milk, in that the principal immune globulin fraction of drying-off secretions (Figure 1E) and of mastitic milk (Figure 4C) is not adsorbed on DEAE, hence elutes with zero NaCl concentration in Peak 1. In contrast, this fraction is quite low in colostrum, the bulk of the immune globulins are adsorbed on the column, and a higher ionic strength is required for elution.

In Figures 6 and 7, it can be seen that the main immune globulin fraction is eluted with 0.075 *M* NaCl. Though the curve is not shown, when 0.05 *M* NaCl was used (corresponding to Peak 2 in Figures 1E and 4C), the major immune

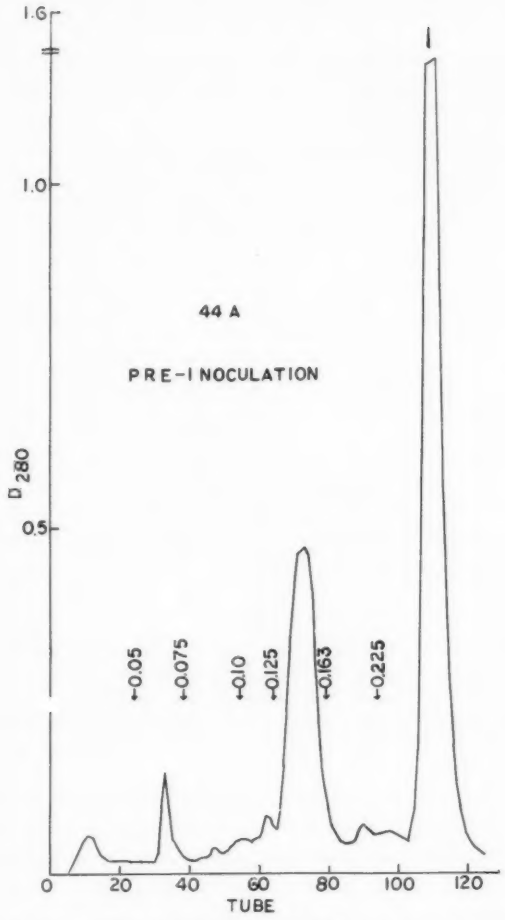

FIG. 4. A, B, and C. Elution diagrams of wheys obtained from Cow 44, Quarter A pre-inoculation (A), 8 hr. post-inoculation (B), and 72 hr. post-inoculation (C) with 4×10^6 *Aerobacter aerogenes* organisms.

(4A) Immune globulins—tubes 6-63, α-lactalbumin, tubes 64-84, blood serum albumin, tubes 85-91, and β-lactoglobulin, tubes 92-130.

globulin fraction eluted with this buffer. The introduction of 0.025 *M* NaCl resolved a peak between the break-through fraction (Peak 1) and the major peak (Figure 7); however, in all cases, four immune globulin peaks were resolved.

The high background of unresolved protein between Peak 1 and blood serum albumin (Peak 6, eluted with 0.163 *M* NaCl) suggests contamination of intervening peaks with the protein of the major immune globulin peak.

Quantitation of the curve for colostral whey from Cow 2481 (Figure 6) indicated the following relative concentrations: immune globulins 71.6%, α-lactalbumin, 6.7%, blood serum albumin 5.5%, and β-lactoglobulin 16.2%, respectively. Peak 2 represented 48% of the total whey protein and was eluted in but four tubes.

Mastitic milk whey proteins. Inoculation of all quarters of Cow 44 with *A. aerogenes* as was mentioned in the Methods section produced varying degrees of response in the four quarters. By 8 hr., the A quarter was hard and inflamed. Milk from this quarter contained many flakes and clots, had a breed cell count of 50,000,000, and produced a strong CMT-3 reaction. The pH was 7.1 and the chloride level of the milk was 0.27%. Milk production from the four quarters dropped from a normal of 10 lb. at the P.M. milking to 6 lb. Milk from A quarter was CMT-negative, had a pH of 6.8 and a chloride level of 0.12%, with no body cells present at zero-hour. At 24 hr., A quarter was dry and only enough fluid for bacteriologic study could be expressed. Total milk

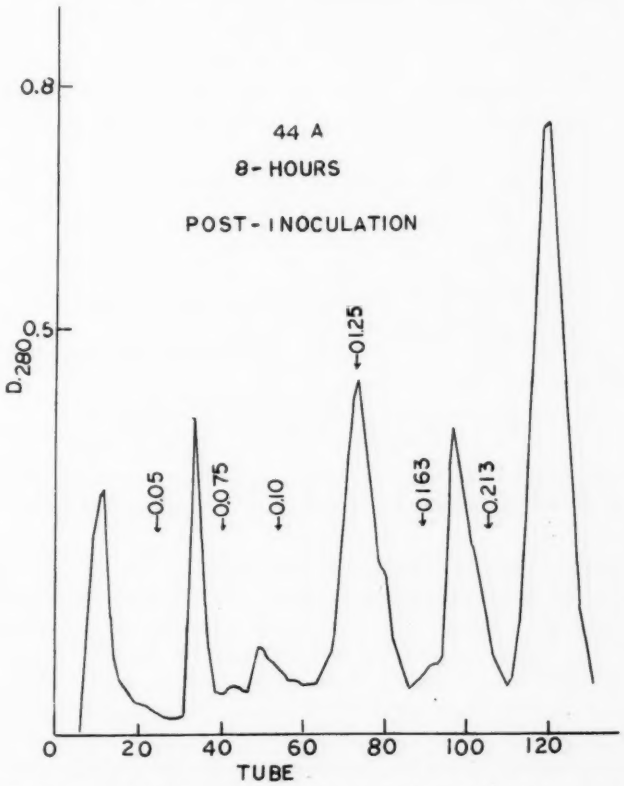

FIG. 4B. Immune globulins, tubes 6-60, α-lactalbumin, tubes 61-97, blood serum albumin, tubes 88-110, β-lactoglobulin, tubes 111-138.

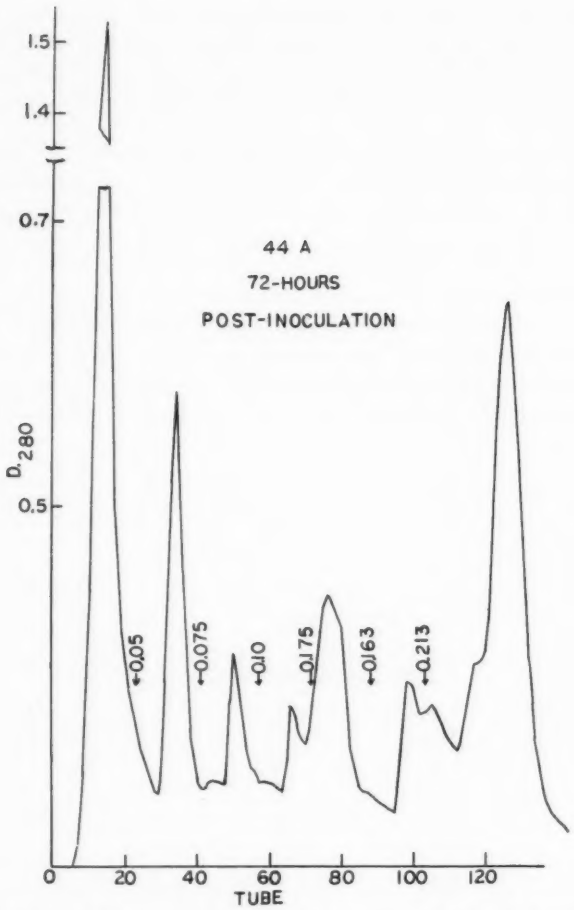

FIG. 4C. Immune globulins, tubes 5-70, α-lactalbumin, tubes 71-95, blood serum albumin, tubes 96-102, β-lactoglobulin, tubes 103-143.

production dropped from a normal of 20 to 6.5 lb. By 72 hr., the total milk production rose to 14.5 lb., though but 250 ml. of milk could be obtained from A quarter. Chloride levels at this time from A quarter were 0.25%, the CMT reaction was still a strong 3, and a breed cell count of 38,000,000 was present.

Elution curves of whey proteins of the preinoculation, 8- and 72-hr. samples are shown in Figure 4. The curves show that by 8 hr., a sharp rise in blood serum albumin had taken place. Relative values at 8 hr. were: Immune globulins, 26.6%, α-lactalbumin, 20.1%, blood serum albumin, 16.9%, and β-lactoglobulin, 36.4%, respectively. Since the possibility existed that one of the β-lactoglobulins might have co-eluted with the blood serum albumin (as can

be seen in Figure 4C), the 8-hr. whey was separated by paper electrophoresis and quantitated on the Analytrol. Values found by this method are indicated in Figure 5. Though blood serum albumin and β-lactoglobulin were not resolved well by this method, the higher serum albumin level indicated the value obtained by DEAE is a good approximation. The 72-hr. relative values were: immune globulins, 51.0, α-lactalbumin, 13.4, blood serum albumin, 8.8, and β-lactoglobulin, 26.8%, respectively. A level of total whey protein of 1.8% was found with this sample. This indicates the milk contained on an absolute basis, 0.92% immune globulins, 0.24% α-lactalbumin, 0.24% blood serum albumin, and 0.48% β-lactoglobulin.

GENERAL DISCUSSION

In agreement with others (9), the immune globulin fraction of whey was found to increase markedly in the drying-off phase of lactation. Quantitatively, these fractions approach those found in colostrum; however, more protein is present in the breakthrough fraction (Peak 1) in drying-off secretions than is present in the same fraction from colostrum. Presumptive evidence suggests a part of the difference may be due to a colored component in higher concentration in this fraction from drying-off milk.

The results suggest that Peaks 1-4 do not represent homogeneous proteins. This is not unexpected, since immune globulins probably represent a continuous

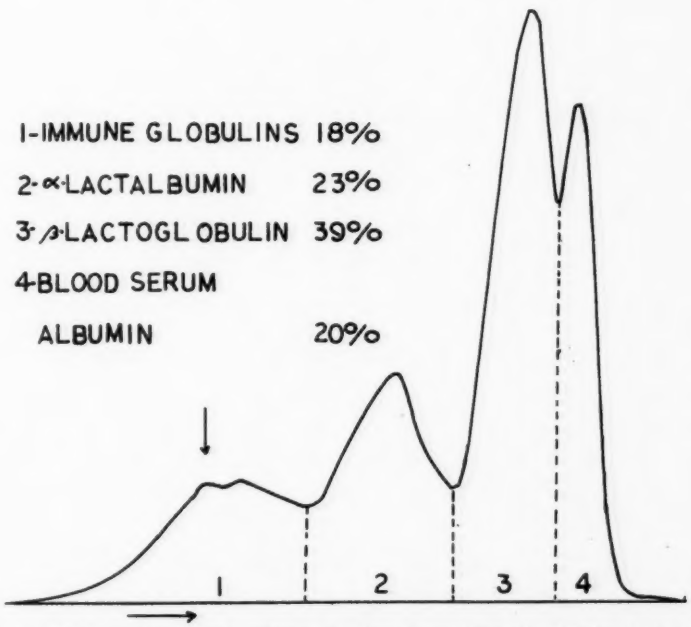

FIG. 5. Paper electrophoresis pattern of whey from Cow 44, Quarter A, 8 hr. post-inoculation with 4×10^6 *Aerobacter aerogenes* organisms.

spectrum of proteins with but slight physico-chemical differences. The fact that the bulk of the immune globulins from colostrum was obtained in four tubes (Figure 6) indicates the suitability of this method for studying the immune properties of milk.

The salting-out experiments indicate the yield of pseudoglobulin and euglobulin is markedly affected by the method of preparation. This was also observed by McDougall (12). Present evidence suggests that DEAE separation bears little relation to the relative solubility of immune globulins in water, i.e., no peak can be said to be principally euglobulin or pseudoglobulin.

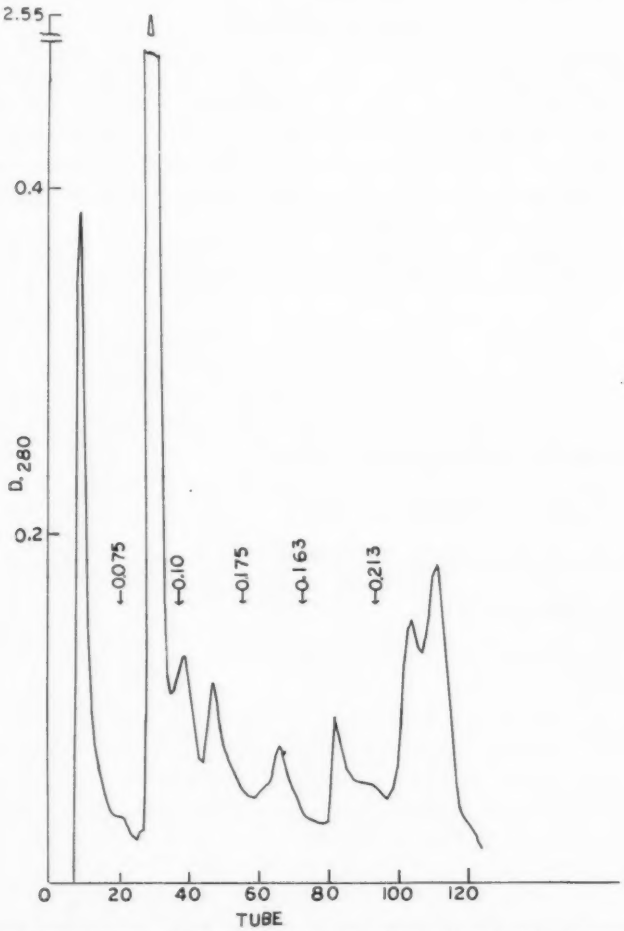

FIG. 6. Elution diagram of whey proteins of colostrum (Cow 2481). Immune globulins, tubes 7-59, α-lactalbumin, tubes 60-80, blood serum albumin, tubes 81-97, and β-lactoglobulin, tubes 98-123.

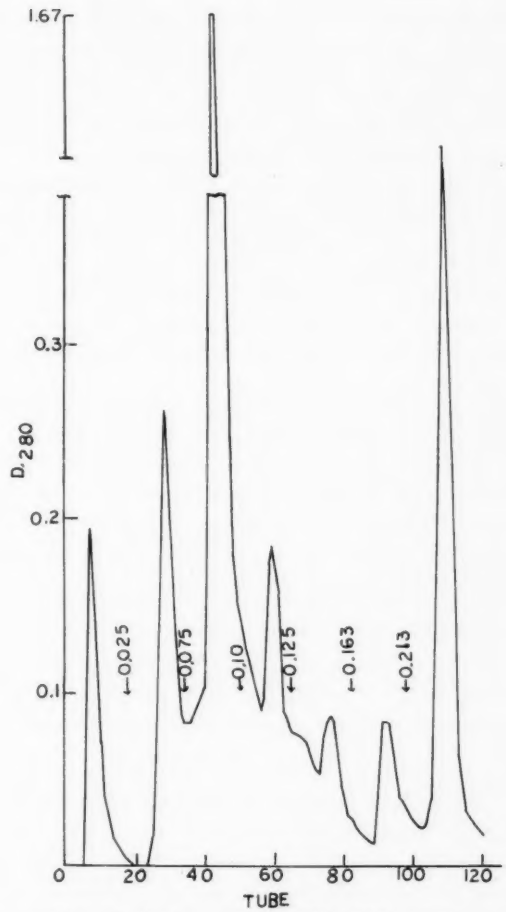

FIG. 7. Whey proteins of colostrum (Cow 2414). This colostrum represents the first milking after that on October 13 (Figure 1E). Immune globulins, tubes 5-73, α-lactalbumin, tubes 74-89, blood serum albumin, tubes 90-103, and β-lactoglobulin, tubes 104-121.

The results presented indicate that following a severe drop in milk production blood constituents including immune globulins, blood serum albumin, leukocytes, and chlorides concentrate. However, during the drying-off phase, the udder-formed whey proteins appear to be produced at a constant rate (Figure 3). Increased chlorides probably reflect decreased lactose. Increased CMT scores parallel increased cell counts whose levels appear to reflect somewhat the lack of dilution with milk rather than to an inflammatory process *sensu stricto*. The high levels of epithelial cells found in the latter drying-off milks (but not reported) suggest the beginning of the involution process (17). The transudation of blood proteins, on the other hand, appears to be more than

passive, since the rate of increase in immune globulins is greater than that of blood serum albumin. The rate of change is inverse to their concentration in the blood and to their relative molecular weights. Values for the 8-hr. mastitic milk (Figure 5) suggest passive leakage and altered permeability, in view of the greater ratio of serum albumin to immune globulins (0.9 as opposed to 0.17 at 72 hr. and 0.24 on October 10 in drying-off milks).

These observations suggest that as milk production drops, there is either (1) local production of globulins (antibodies), (2) selective resorption of blood serum albumin, or (3) a selective transudation of globulins from blood to milk. Although plasma cells are believed to be the site of antibody formation (7), and these cells are reported to be present in abundance during colostrum formation and after cessation of milking (2), antibody production without an apparent antigenic stimulus can not be rationalized. Bacteriologic examination of the drying-off secretions showed them to be sterile. Though resorption from the udder has been demonstrated (16, 17), the selective transudation of immune globulins appears to be the more probable mechanism (3, 4).

With the use of immunohistochemical procedures in the hyperimmune animal, Dixon and coworkers (3) found the udder to concentrate immune globulins. Coupled with phase microscopic observations of udder tissue throughout lactation (4), these workers in contrast to Campbell *et al.* (2), noted that plasma cells were not particularly prevalent during colostrum formation and pointed out that interpretation of histologic material may account for the discrepancy. Of most significance relative to present work, convincing evidence was presented to show that the acinar cells of the udder epithelium were the active sites in the selective removal of gamma globulins from the blood. It is these cells that appear to be the first to undergo cytological change in mastitis (5).

The difference found between the immune globulins of the drying-off secretions and colostrum suggests that if colostrum is but an accumulation during the dry period (8), either some mechanism exists favoring transudation of component(s) of Peak 2 (Figure 6) over those of Peak 1 (Figure 1E) during the dry period, or, prolonged stay in the udder brings about physico-chemical changes imparting stronger affinities of the protein(s) for DEAE. Experiments in progress on dry-cow secretions have shown that Peak 1 still predominates 1 mo. in the dry period.

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CONCENTRATIONS AND PERCENTAGE RECOVERY OF FURACIN IN MILK FOLLOWING INTRAMAMMARY INFUSIONS[1]

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SUMMARY

Four experiments involving 106 quarters of the udder of dairy cows were carried out to determine the rate and cause of disappearance of furacin from milk following intramammary infusions.

In Trial 1, milk from 43 quarters contained only traces (< 0.5 p.p.m.) of this antibacterial agent at the first (14 hr.) and second (24 hr.) post-infusion milkings. In subsequent trials it was found that the mean concentration and mean percentage recovery of furacin in milk decreased at a rapid rate as the interval between infusion of the drug and milking out of the gland was lengthened. Concentrations and recoveries of the drug were highest in milk withdrawn 1 to 2 hr. post-infusion. Rate of disappearance of furacin in milk varied with individual cows. A spectral analysis of milk filtrates did not yield positive evidence that furacin infused into the mammary glands was metabolized to reduced furacin, as had been reported for in vitro incubations with other biological preparations. It is postulated that the disappearance of detectable furacin in milk from infused quarters is due to the absorption of the drug or its metabolites into the blood stream or the formation of a chemical complex between furacin and milk proteins.

Furacin (5-nitro-2-furaldehyde semicarbazone) is an antibacterial drug used in the dairy industry in the treatment of mastitis, uterine and topical infections, and as an antidiarrheal. Most drugs infused into the udder are secreted in milk from the infused quarter for varying periods of time. Milk containing drug residues can not be marketed legally. Therefore, the amount of time that must elapse between administration of antibacterial drugs to cows and the production of milk free of drug residues is important to dairymen.

A study was made to determine the rate and cause of disappearance of furacin in milk from quarters of the udder of cows infused with this drug.

EXPERIMENTAL PROCEDURE AND RESULTS

Furacin was infused into 106 normal quarters of udders of lactating cows. Each medicated quarter of the udder was infused with 60 mg. furacin (a 30-ml. squeejet of 2.0% furacin, in a water-miscible vehicle). Infusions of the drug were made at 1 to 14 hr. before milking, as indicated for the four trials.

Experimental cows used in this investigation included purebred Holsteins and Jerseys and Holstein-Jersey crossbreds in early to late stages of lactation. Total daily milk production of individuaal cows ranged from 11.7 to 57.2 lb. The cows were milked either by hand or by quarter-milkers. Milk from individual quarters of the udder was collected separately, weighed, and an aliquot taken for analysis to determine furacin concentration.

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The concentration of furacin was determined spectrophotometrically within 4 hr. after milking. In Trials 1, 2, 3, and 4 the method described by Paar (4) was used, whereas in the preliminary phase of Trial 2, furacin was determined by the method reported by Buzard *et al.* (3). Both methods have a similar degree of sensitivity, with the lower limit of accuracy being approximately 0.5 p.p.m. However, the method described by Paar (4) is more suitable than the alternate method (3) for routine determinations on a large number of samples. Basically, this method (4) used for routine determinations consisted of precipitation of milk proteins with sulfuric acid and sodium tungstate, followed by centrifuging and filtering to obtain a milk filtrate. Concentration of furacin in the filtrate was then determined spectrophotometrically at 375 mμ wave length.

Trial 1. Four quarters of the udders of ten cows and the three functional quarters of the udder of another cow were each infused with furacin immediately after the afternoon milking. All cows were milked at 14 hr. and again at 24 hr. post-infusion. Milk from each of the 43 quarters infused with 60 mg. of furacin contained less than 0.5 p.p.m. of this bactericidal drug at the 14- and 24-hr. post-infusion milkings.

Since the concentration of furacin was below the level of accuracy of the method, the exact concentration could not be determined. However, results of this investigation show that concentration of furacin in milk was extremely low at 14 and 24 hr. post-infusion. One of the infused quarters from the lowest-producing cow yielded only 1.0 lb. of milk and an infused quarter from the highest-producing cow yielded 12.0 lb. of milk at the 14-hr. milking. Assuming 100% recovery in the first milking, concentration of furacin in milk from the lowest-producing quarter would have been 132 p.p.m. and that from the highest-producing quarter, 11 p.p.m. Milk from both of these quarters contained no detectable furacin at the 14-hr. milking.

Trial 2. To study the rate of disappearance of furacin from milk, the interval between infusion of furacin into the quarter and milking out of the quarter was shortened. In the preliminary phase of this trial, each quarter of the udder of two cows was infused at 7 A.M., approximately 1.5 hr. after they were milked. One quarter was milked out at 8 A.M. (1 hr. post-infusion); other quarters were milked out at hourly intervals, with the eighth quarter being milked out at 8 hr. post-infusion.

In the main phase of Trial 2, the approach differed from that of the preliminary investigation, in that the intramammary infusions were made at different intervals before milking and the cows were milked at the regular milking period. At the time of infusion, the quarters contained the milk manufactured since the last regular milking. Six cows (three Holsteins and three Jerseys) producing 17.6 to 49.6 lb. of milk daily were used as experimental subjects. On each of three successive days, the left fore and left rear quarters of the udder of each cow were infused with furacin at 2.0, 3.5, or 4.7 hr., and a few days later at 7.8 hr. before the scheduled milking period. The actual milking time varied as much as 0.5 hr. from the schedule. Milk withdrawn from individual

quarters at the first and second post-infusion milkings was analyzed for furacin content. Since there was some variation between the actual and scheduled milking time, all furacin concentration and recovery data were adjusted graphically to the interval given in the schedule, to facilitate statistical analysis. A statistical study of the data shows that the variance of the mean at each post-infusion interval was not equally distributed. Therefore, an arc sine transformation (5), more nearly equalizing the distribution of variance, was made before analysis of variance of the data. Subsequently, the data were retransformed for the purpose of interpretation.

Volume of milk produced, concentration of furacin in milk, and percentage recovery of infused furacin at hourly intervals, during the preliminary phase, are presented in Table 1. The highest concentration of furacin was detectable

TABLE 1

Volume of milk produced, concentration of furacin in milk, and percentage recovery of the infused drug at hourly intervals

Hours post-infusion	Cow and quarter	Volume of milk	Furacin concentrations	Furacin recovered
		(ml.)	*(p.p.m.)*	*(%)*
1	(1) LR	165	19.9	5.64
2	LF	330	11.8	6.71
3	RF	800	2.4	3.33
4	RR	860	1.9	2.87
5	(2) LR	540	1.4	1.26
6	LF	434	4.4	3.26
7	RF	467	1.0	0.78
8	RR	768	0.6	0.77

in milk withdrawn 1 hr. post-infusion, with a rapid drop in the concentration of this drug in milk withdrawn at 2 and 3 hr. post-infusion. Concentrations of furacin in milk from quarters milked out at subsequent hourly intervals continued to decrease, except for an increase in the milk from the quarter milked out 6 hr. post-infusion. Percentage recovery of the infused drug in milk was highest at 2 hr. and lowest at 8 hr. post-infusion. The difference in percentage recovery of the drug in milk at the 1- and 2-hr. post-infusion intervals could have been due to a higher ratio of residual milk retained in the quarter at the 1-hr. than at the 2-hr. interval.

Mean concentrations and mean percentages of recovery of furacin in milk at the first milking after infusion into the quarters at different premilking intervals (main phase, Trial 2) are presented in Table 2. Highest mean concentration and the highest percentage recovery of furacin in milk was at the 2-hr. post-infusion milking. Concentrations of detectable furacin and percentage recovery of this drug in milk at the 2.0-, 3.5-, and 4.7-hr. post-infusion milkings differed significantly ($P < 0.05$), according to Student's "t"-test (5). However, the mean concentration and mean percentage recovery of detectable furacin in milk did not change significantly ($P > 0.05$) from the 4.7-hr. to the 7.8-hr. post-infusion milkings. Variations in concentrations of furacin in milk with-

TABLE 2

Relationship between interval that furacin remained in the quarter and concentration of the drug in milk and the percentage recovery

Time drug was in quarter	Concentration in milk			Recovery of drug		
	Mean	Confidence limits[a]		Mean	Confidence limits[a]	
		Lower	Upper		Lower	Upper
(hr.)	*(p.p.m.)*			*(%)*		
2.0	8.7	5.7	14.4	21.7	14.2	30.1
3.5	2.4	1.0	4.5	6.4	2.5	11.9
4.7	0.6	0.1	1.8	1.2	0.0	4.2
7.8	0.3	0.0	1.1	0.5	0.0	2.8

[a] Retransformed from arc sine transformation (5).

drawn at the same post-infusion periods were large, as shown by the confidence limits. Some of the variation in concentration at a given post-infusion period was associated with breed. The mean concentration of furacin in Jersey milk was significantly greater ($P < 0.01$) than that in Holstein milk. Apparently, some of the variation in furacin concentration associated with breed was related to level of milk production, which averaged 37% greater for Holsteins than for Jerseys. This is indicated by the highly significant correlation ($r = -0.485$) between levels of milk production and concentrations of furacin in milk. In addition, a significant percentage of the variation in concentration of furacin in milk was associated with individual cows ($P < 0.01$). However, there was no significant difference in concentration of furacin in milk between the front and rear quarters of the cow ($P > 0.05$).

There was no significant relationship between the percentage of the infused drug recovered in milk and the breed of cow or the level of milk production.

Concentration of furacin in milk from 42 quarters was determined at the second post-infusion milking. The second post-infusion milkings were from 16 to 18.8 hr. after infusion with furacin. Milk from 35 of the quarters contained no detectable furacin, whereas that from the remaining quarters contained traces of furacin, less than 0.5 p.p.m. and estimated at less than 0.2 p.p.m. in all samples.

Trial 3. In vitro studies were made in which furacin was added to ten raw milks and incubated at 37-40° C. for 6 to 23 hr. In the first phase, 10 p.p.m. of furacin was added to two milks and aliquots taken for analysis at 4, 6, and 22 hr., to study the rate of disappearance of the drug. Recovery of the added furacin was 85% or more through the 6-hr. sampling period, but recovery decreased to 0% at the 22-hr. period. In the last phase of this trial, 24 p.p.m. of furacin was added to milks from eight cows and incubated at 37° C. for 23 hr. Recovery of furacin after 23 hr. of incubation ranged from 86.3 to 93.8%. Difference in recovery of the drug after 22 to 23 hr. possibly resulted from the small differences in temperatures. Nevertheless, the high percentages of recovery of added furacin after incubation, in vitro, at 40° C. for 6 hr. and at 37° C. for 23 hr., were larger than recovery of the drug in milk at similar periods after intramammary infusions.

Trial 4. Studies by other investigators (1, 2, 6) have shown that incubation of furacin with different biological preparations produced a shift of the peak of maximum absorption of light. The absorption maximum of furacin at 375 mμ disappeared with the development of a peak at 335 mμ because of reduced furacin (1, 2, 6) and at 290 mμ resulting from production of uric acid (2, 6). To determine whether the same breakdown occurred in the udder, seven additional quarters of udders of four cows were infused with furacin. Milks from these quarters were used to prepare filtrates for a study of the absorption spectra of milk filtrates from furacin-infused quarters, as compared with those of milk filtrates from noninfused quarters. Furacin remained in the quarters for intervals ranging from 1.1 to 6.6 hr. Within 5 min. after withdrawal of the milk, filtrates were prepared using the sulfuric acid–sodium tungstate precipitation procedure (4). Subsequently, the absorption spectra of these filtrates were determined as rapidly as possible with a Model DU Beckman spectrophotometer over the range 320 to 400 mμ at 5- to 10-mμ intervals. Interfering substances in the milk filtrates prevented measurements of optical density below 320 mμ.

Maximum absorption of light by the milk filtrates resulting from 1.1- and 1.9-hr. post-infusion milkings occurred at the 375-mμ wave length only. There was no detectable peak absorption of light by these filtrates at 335 mμ, as had reportedly occurred when furacin was incubated with other biological preparations in vitro (1, 2, 6). Filtrates from milk withdrawn at other post-infusion intervals did not give a detectable peak absorption within the 320 to 400 mμ wave length range. However, the absorption curves of four out of eight milks incubated, in vitro, at 37° C. for 23 hr. after furacin was added (Trial 3), showed a distinct shoulder at 335 mμ but retained the 375-mμ peak.

To avoid the interference in the milk filtrate absorption spectra associated with reagents used previously, a milk filtrate was prepared by precipitation of the protein with trichloroacetic acid. The absorption spectra of a furacin-free milk filtrate was compared with that of a milk filtrate prepared from milk withdrawn 1 hr. after furacin was infused. Optical densities of the filtrates were measured against a distilled water blank over the wave length range of 250 to 420 mμ. Both the control milk filtrate and the furacin-milk filtrate absorbed some light at all wave lengths. Absorption of light by the furacin-milk filtrate was greater than that of the control filtrate at all wave lengths measured at 5 to 10 mμ intervals between 310 and 420 mμ; however, from 310 to 250 mμ, the optical density of the control milk filtrate was similar to that of the furacin-milk filtrate. The difference in optical density of the control filtrate and the furacin-milk filtrate indicated that the drug contributed to light absorption over the 310 to 420 mμ range only. A peak absorption of light by the furacin-milk filtrate occurred at 375 mμ, but there was no detectable peak at 335 mμ.

DISCUSSION

Concentration of detectable furacin and the percentage recovery of this drug in milk following intramammary infusion decreased as the interval between

infusion of the drug and milking out of the quarter was lengthened. For any given interval between infusion of the drug and milking out of the quarter, there was a negative correlation between level of milk production and concentration of the drug in milk. Also, it was found that there was a highly significant difference ($P < 0.01$) between cows in the percentage of recovery of furacin in milk from infused quarters. The highest percentage of infused furacin recovered at the 2-hr. post-infusion milking was 42.0, and average percentage recovery at that interval was only 22.5. At the 7.8-hr. post-infusion milking, average recovery of furacin was only 1.2%. When the first milking occurred more than 8 hr. post-infusion, concentration of furacin in milk and percentage of recovery were too low to determine with a measurable degree of accuracy.

Decreases of detectable furacin in milk following intramammary infusions, as indicated by disappearance of the absorption maxima at 375 mμ wavelength, were not accompanied by the development of absorption maxima at 335 mμ resulting from reduced furacin, as reported for other biological preparations (1, 2, 6). Conversely, the absorption curves of filtrates prepared after in vitro incubation of furacin in milk showed a shoulder at 335 mμ, which suggested the presence of reduced furacin.

There appears to be two plausible explanations for the rapid decrease of furacin concentration with lengthened post-infusion intervals. They include: (a) rapid absorption of furacin or its metabolites into the blood, or (b) rapid formation of a chemical complex between furacin or its metabolites and the proteins in milk. Blood samples collected from two cows at approximately 1 hr. post-infusion, when compared with a filtrate from a nontreated (control) animal, showed no positive detection of furacin, since the optical densities of blood serum filtrates from all animals were quite high. However, this does not preclude the presence of the drug in the samples, since protein complexes may have developed.

Further studies involving radioisotopes are needed to determine the mechanism by which furacin disappears from milk following intramammary infusions.

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PREPARATION, ASSAY, AND PRELIMINARY CHARACTERIZATION OF BOVINE GASTRIN

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SUMMARY

A gastrin-like gastric secretory stimulant was isolated by an alkaline extraction method from pyloric and fundic mucosae of the bovine abomasum. Secretory activity was measured after subcutaneous injection into Gregory and Heidenhain pouch dogs. The injected material was shown not to be histamine. The reaction to bovine gastrin is usually delayed for 60 to 90 min., in contrast with porcine gastrin, which reacts within 30 min., and with histamine, which reacts in even less time. The most active fractions of bovine gastrin were obtained by isoelectric precipitation at pH 3.0-4.0, in contrast with porcine gastrin, which has highest activity in the fraction precipitated at pH 5.0-5.5. The response to bovine gastrin, as is the case with the porcine material, persists for from 2 to 4 hr., contrasting with the speedy decline after response to histamine. In preliminary purification, we found a nondialyzable fraction, perhaps conjugated with or tenaciously held by a larger molecule, and a dialyzable fraction. Partial purification by ion-exchange has been effected. Preliminary chemical analysis shows that crude bovine gastrin is very similar to crude porcine gastrin in IR and UV spectra and phosphorus content.

Gastrin is the name given to the hormonal stimulant or stimulants of gastric secretion. It presumably acts by effecting the release of histamine at the parietal cell which, in turn, causes an increased flow of strongly acid, clear gastric juice. The presence of such a hormone-like substance has been well established. Babkin (4) reviews the work on gastrin up to recent years. It has been isolated chiefly from antral mucosa of animals with simple stomachs (especially the hog) and to a slight extent from human stomachs (17). Recently, a gastrin-like material has been found in pancreatic tumors of patients suffering from the Zollinger-Ellison syndrome (10), and a bovine pancreatic tumor has also been reported (16).

In the course of extensive work in this laboratory, aimed at purification and identification of the active factor or factors, it was of interest to examine the stomach mucosae of several species of animals. Bovine abomasal tissue seemed of particular interest, because of differences between the digestive physiology of ruminants and animals such as the hog and dog (2, 3). In ruminants, a relatively constant flow of food material through the abomasum (12a) would seem to require a steady release of free acid and, therefore, constant stimulus

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by gastrin, which it was thought might be isolable in relatively large amounts. Furthermore, since the pH of the acid secretion in ruminant stomachs is never as low as is common in animals with simple stomachs, the gastrin from these species might have different properties or might be attached to a different type of molecule.

Relatively little seems to be published on the physiology of the bovine and ovine abomasum or true stomach (1-3, 13, 14), although considerable research has been reported on the physiology of the rumen of these species (7). The mechanism of acid secretion by animals with simple stomachs has been divided into three phases: psychic, gastric, and intestinal. These, separately or together, in a complex and much-studied process, elicit release of strongly acid secretion from the parietal cells. It is not known whether the subsequent decline in strongly acid secretion is caused by an inhibitory hormone or merely results from inhibition of the release of gastrin by the flow of acid secretion over the antrum.

In addition to the continual flow of food material into the fourth stomach, the abomasum differs from the simple stomach in that there is a pronounced pH gradient, ranging from pH 6.0 or above at the cardial end to pH $\sim$ 2.0 at the pyloric end (3). It is reasonable to assume that psychic secretion in the ruminant is not important, especially in relation to the synchronizing of ingestion of food and the production of gastric acidity, since it has been proven that the rate of passage of the digesta through the abomasum of sheep regulates the flow of acid (3).

Several methods have been reported for the preparation of gastrin (4). Gregory *et al.* have recently reported a simple picric acid method and a further more complex procedure (10), and this laboratory has given a preliminary description of a new alkaline extraction method (8).

METHODS

Source and preparation of bovine gastrin. Gastrin was extracted by a modification of our published method (8), from antral and fundic mucosae stripped from the abomasum of beef and dairy cattle at the time of slaughter at a local abattoir.

The tissue was cleaned of food particles and adhering fat, placed in freezer bags, and taken to the laboratory at about 25-30°. The extraction procedure was started within 2 hr. of slaughter. The tissue was soaked in acetone for a half-hour, drained, air-dried, and minced in a meat grinder.

The ground tissue was then stirred into three liters of water at 40° C. and the mixture made alkaline (pH 10.5—pH meter) by gradual addition of *N* sodium hydroxide. After stirring for 3 hr., the mixture was filtered through cheese cloth and the filtrate adjusted to pH 7.0 with *N* hydrochloric acid, and the resulting precipitate was centrifuged off and discarded. The supernatant solution was then adjusted to pH 4.0 (*N* HCl) and the precipitate centrifuged off, washed three times with acetone and once with ether, and air-dried. This precipitate was then taken up in aqueous sodium hydroxide (pH 10.5) and the

clear solution again acidified, by the addition of hydrochloric acid, to pH 4.4. After centrifuging and drying, as before, the powder was gently and thoroughly ground in a large mortar with three liters of 0.9% sodium chloride solution held at 40° C. The suspension was stirred 30 min. and the pH adjusted (*N* NaOH) to 8.0. Stirring was continued for another 30 min. at 40° C. Insoluble material was then centrifuged off and discarded and the supernatant brought to a pH of 5.0. In our preparations of porcine gastrin, the precipitate at this stage is biologically active, but we found little if any activity in material at this step in the bovine preparation.

Further precipitates of the bovine material were collected at pH 4.0 and 3.0. The latter consistently initiated gastric secretion. The supernatant solution from this step may be lyophilized, or a further precipitate obtained by addition of several volumes of acetone. Both methods have produced biologically active material.

All precipitates were taken up in aqueous alkali (pH 11.0) and magnetically stirred and dialyzed for five days against distilled water changed daily. These fractions, which reprecipitated from the solution within the sacs, were dried and stored in a deep freeze. The dialyzates were collected, concentrated at 40° C., and lyophilized.

Dialyzates. Dialyzates of isoelectric precipitates were desalted by reprecipitation at pH 3.0, or by taking up on an Amberlite IRC-50 acetic acid column by the method of Dixon (6). The eluate containing the secretory stimulant was lyophilized to remove acetic acid and stored as a powder.

Phosphorus. Phosphorus was determined by slight modification of the method of Hirata and Appleman (12).

Histamine. Histamine was assayed by Dr. Akira Horita of the Department of Pharmacology, using the fluorometric method of Shore, Burkhalter, and Cohn (15).

Ultraviolet and infrared spectra. Ultraviolet absorption spectra were run on a Beckman DK-1 spectrophotometer. Samples of the secretory stimulant were dissolved in mildly alkaline water solutions to a concentration of 300 μg. per milliliter and adjusted to the desired pH just prior to scanning.

Infrared absorption spectra were determined on a Beckman IR-5 spectrophotometer, using ¾ mg. of gastrin preparation per 300 mg. KBr.

Heidenhain and Gregory pouch preparation in the dog. Healthy mongrel dogs with Heidenhain or Gregory (9) denervated gastric pouches were used for the assay of gastrin activity.

In the Heidenhain pouch, a standard preparation for the assay of gastric secretory activity, the vagus nerve fibers are cut. The Heidenhain pouch has the disadvantage, however, of having intact sympathetic nerve fibers. The Gregory pouch is a modification of the Heidenhain pouch, constructed by interrupting the sympathetic nerve fibers as they pass along the splenic artery to the portion of the greater curvature which will form the pouch, and by interrupting the gastroepiploic artery with its accompanying sympathetic nerve fibers at the point where it would pass into the inferior portion of the pouch.

In addition, the spleen is removed, eliminating its shunting effect and insuring a more constant blood flow to the pouch. During the operation epinephrine (10 ml. of 1 : 10,000 solution) is injected into several of the small splenic artery branches, to cause the spleen to contract and empty most of its contained blood into the systemic circulation. The pouch blood supply is derived wholly from the short gastric branches of the splenic artery, which are preserved by ligating the multiple small branches of the splenic artery and vein just as they enter the spleen. The remainder of the procedure is identical with the preparation of a Heidenhain pouch (5). The Gregory pouch is an attempt to obtain complete denervation while retaining a blood supply adequate for a significant level of secretion. These completed preparations are shown diagrammatically in Figure 1.

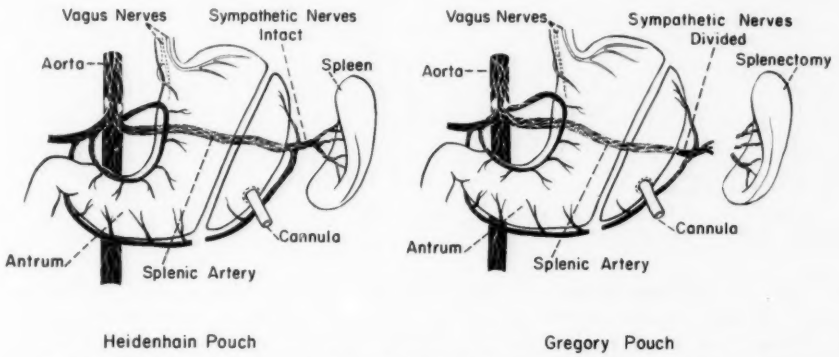

FIG. 1. Diagrammatic preparation of Heidenhain and Gregory pouches in dogs.

Bioassay. For animal assay, material from the various steps of our prepara- was dissolved in slightly alkaline (pH 10-11) 0.9% sterile saline, and the pH carefully lowered to 6.8-7.4 (*N* HCl). The solutions were injected in different areas subcutaneously, all at the same time in four equal portions, into pouch dogs previously fasted for 24 hr. A baseline secretion was collected for at least 15 min. prior to injection. Collections were made every 15 or 30 min. in 15-ml. graduated tubes. Free acid was determined by titrating to pH 3.3 with 0.01 *N* NaOH. pH change was determined with pHydrion papers.

RESULTS AND DISCUSSION

Dog assay. The physiological activity of bovine gastrin thus injected into Heidenhain and Gregory pouch dogs was similar to that of porcine gastrin (see Figure 2). In general, we found the response, after injection of bovine gastrin, delayed at least 30-60 min. more than after injection of porcine gastrin. This might be due to a species difference in the structure of gastrin or to association of the bovine gastrin with a larger (more slowly absorbed) carrier molecule, or to some unknown factor.

The most active fractions of crude bovine gastrin were precipitated at pH

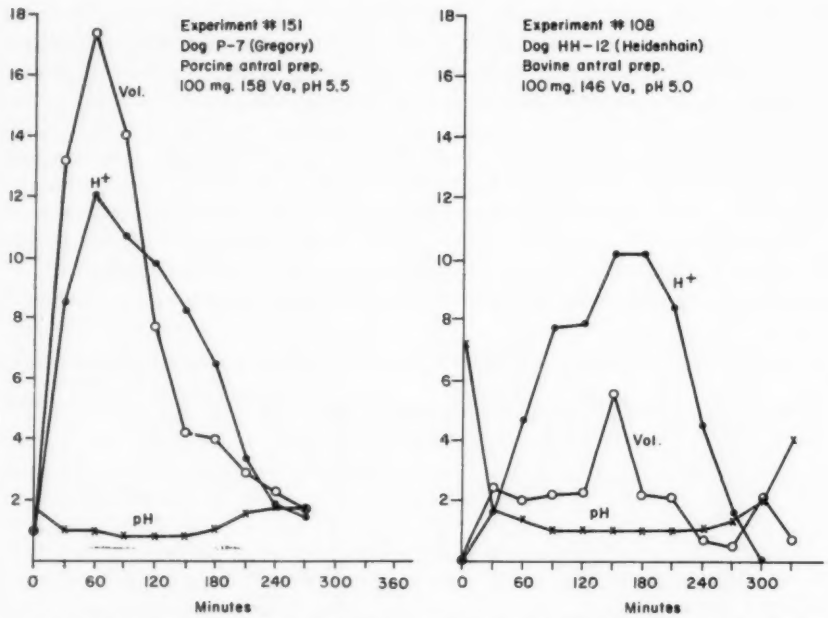

FIG. 2. Comparison of effect of subcutaneously injected gastrin from porcine and bovine antral prepaarations. Units: The volume in Figures 2-5 is expressed as milliliters for the preceding 30 min., and the H^+ is expressed in milliliters of 0.01 *N* NaOH to titrate 1.0 ml. of the collected secretion from that period to pH 3.3. All injections took place at zero time.

3-4. This contrasts with the material isolated from the hog antrum, which is most active from isoelectric precipitation at pH 5.0-5.5.

Figure 3 illustrates the different reactions of the dog to histamine and to bovine gastrin. The response to histamine usually reaches a peak about 30 min. after injection, then subsides rapidly to the baseline in about 60 min. This sample of bovine gastrin (isoelectrically precipitated at pH 3.5) caused no appreciable increase in free acid, volume, or pH until 90 min. after the injection. The full effect was not apparent until 3 hr. had elapsed, and this persisted for more than four more hours.

Figure 4 shows the results of a 50-mg. and a 20-mg. injection of bovine gastrin into different dogs. The material injected in Experiment 167a was obtained by lyophilizing the solution which remained after centrifugation of the pH 3.5 precipitate. The secretory stimulant of Experiment 197 was prepared by desalting dialyzates of isoelectric precipitates on an ion-exchange column, eluting with acetic acid. Again, it is apparent that bovine gastrin preparations maintain a prolonged stimulus as in Experiment 167, in contrast with the rapid decline after response to histamine, but similar to the prolonged effect of porcine gastrin, although the onset of response to the latter is generally much less delayed.

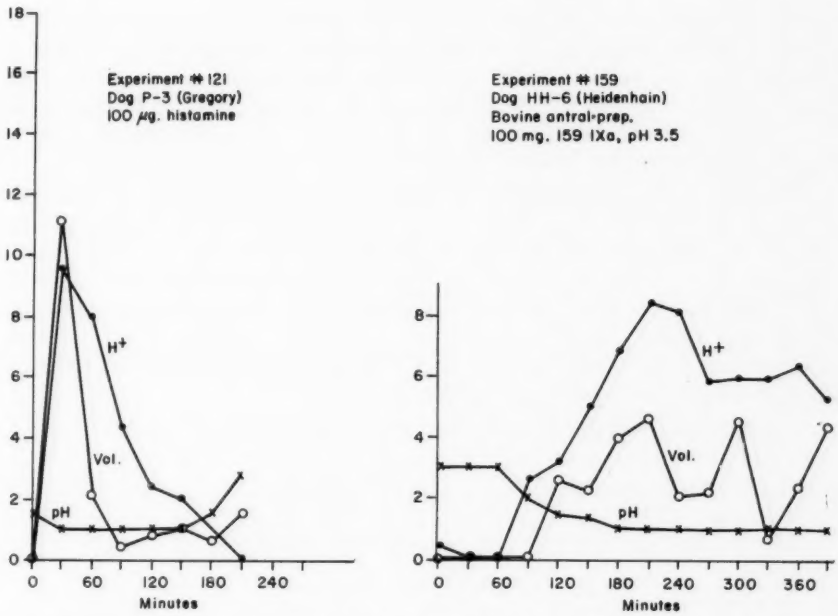

FIG. 3. Comparison of response to histamine and to a bovine antral preparation.

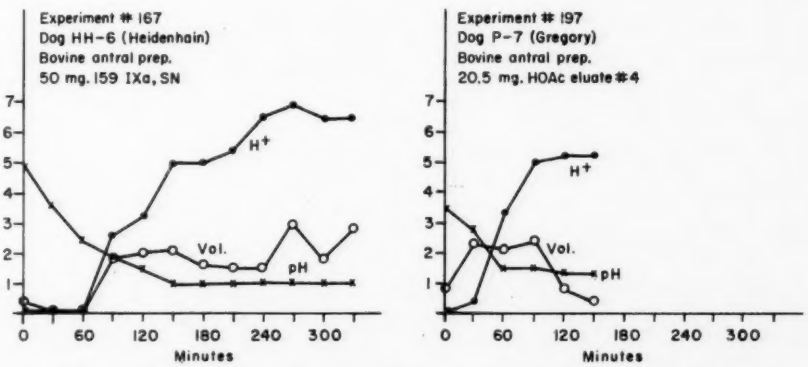

FIG. 4. Response to crude bovine antral preparation and to a dialyzate.

Bovine fundus preparations are compared with a 10-mg. bovine antral extract (Figure 5). The fundus (mucosal) extracts were prepared in exactly the same manner as described above. The preparation in Experiment 193 was taken from the same stomachs as the antral preparation in Experiment 187, Figure 5. Previous attempts to isolate a gastrin-like secretory stimulant from the fundus in simple-stomached animals have not been successful. Recently,

Gregory (10) reported inability to extract gastrin activity from the corpus region of the hog stomach by his new method, but isolated a small amount of gastrin from the cardial region. The bovine fundic preparations have a lower specific activity (degree of stimulation per unit weight) than the bovine antral extracts. This is also true for extracts of fundic mucosae of sheep, according to preliminary findings in this laboratory.

Dialyzates. Gastrin has been reported to be both dialyzable and nondialyzable. In the preliminary report on our new method for preparing porcine gastrin, we discussed only the relatively large molecular weight material, after dialysis. In a forthcoming paper we shall discuss this further and also our experience in obtaining very active fractions after prolonged dialysis of the crude precipitates, as well as finding appreciable activity in the residual dialyzand. In our preliminary work in the attempted purification of bovine gastrin, it appears that the same pattern occurs.

Phosphorus. We had noted a small but consistent content of phosphorus in our samples of porcine gastrin (0.3-0.8%). It is of interest to note that bovine gastrin also contains phosphorus. The material injected in Experiment 159 (Figure 2) contained 1.93% phosphorus; that of Experiment 197, 0.108% (Figure 3), and there was 0.105% phosphorus in the gastrin injected in Experi-

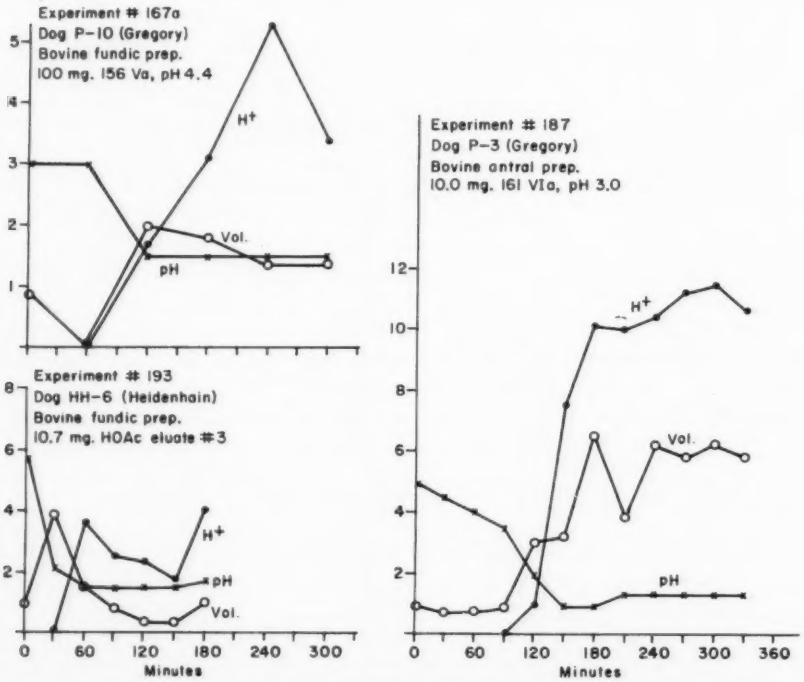

FIG. 5. Response of dogs to bovine fundic and antral preparations.

ment 193 (Figure 4). We suspect that the phosphorus is associated with a nucleotide-like component, as we reported for crude porcine gastrin (8).

Histamine. All of the samples of bovine gastrin that were assayed fluorometrically contained less than 7.0 μg. of histamine in the amount of material injected into the dog. We have found in repeated experiments (subcutaneous injection) that, even for our dogs that secrete most copiously, at least 20 μg. of histamine is required to elicit even a minor change in the pH of the gastric secretion. No appreciable change in volume has been noted when less than 40 μg. were injected. Even more characteristic is the prolonged response to gastrin, in contrast with the speedy decline after histamine.

Ultraviolet and infrared spectra. The precipitates of bovine gastrin exhibited strong absorption in the ultraviolet range at 258-262 mμ. In contrast, the desalted dialyzates showed absorption at 270 mμ. These values are very similar to those obtained for porcine gastrin. Infrared spectra of bovine gastrin are similar to those of the porcine preparation. We feel that at this stage of purification nothing definite can be concluded from either type of spectrum, but we intend to study both types as we purify gastrin.

Since all of these preparations represent gastrin in a crude state and as a (probably) small component of an unknown mixture, it is impossible to say yet whether bovine gastrin is more or less active in the dog than porcine gastrin. In addition to delayed response, and possibly, more prolonged effect, it is notable that definite activity has been found in bovine fundal mucosa as well as in the antrum.

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ROLE OF PREGASTRIC ESTERASE IN THE ABOMASAL HYDROLYSIS OF MILK FAT IN THE YOUNG CALF[1, 2]

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SUMMARY

The hydrolysis of milk fat in the abomasum of the young calf was studied. Whole milk either was nipple-fed (oral feeding) or was placed directly into the abomasum (abomasal feeding). The purpose of the latter system was to minimize the entrance of pregastric esterase. At 1, 2, and 3 hr. after feeding, the contents of the abomasum were sampled and analyzed for total free fatty acids (FFA). The FFA from the oral-fed samples were also separated into three fractions: butyric, valeric, and higher fatty acids. The mean concentrations of total FFA ($\mu M/5$ g. contents) were as follows for the oral and abomasal systems of feeding, respectively: 1 hr., 86, 19; 2 hr., 101, 20; and 3 hr., 120, 30. For the oral-fed samples, the mean concentrations of butyric, valeric, and higher fatty acids were as follows, respectively: 1 hr., 34, 7, 53; 2 hr., 19, 4, 77; and 3 hr., 11, 3, 119.

Previous investigations of pregastric esterase in the young calf have included origin (8), substrate specificity (1-3, 9), and factors that affect its secretion (10, 12, 14). The extent to which pregastric esterase is involved in gastrointestinal lipolysis, however, has not been ascertained. Thus, an investigation was initiated to study the participation of this enzyme in the abomasal hydrolysis of milk fat.

EXPERIMENTAL PROCEDURE

The abomasum was selected for study because it is the first part of the alimentary tract in which pregastric esterase has an opportunity to effect appreciable hydrolysis. Secondly, gastric lipase, if secreted by the calf, is the only other lipolytic enzyme likely to be involved in this area.

General procedures. Two Holstein calves, rumen-fistulated at 3 wk. of age, were used in this study. Both animals were restricted to a diet of whole milk. During the experimental period, starting at 15 wk. of age, two special feeding procedures were employed. Raw whole milk, containing approximately 3.5% fat, either was nipple-fed (oral feeding) or was permitted to flow by gravity directly into the abomasum (abomasal feeding) via a small rubber tube inserted through the rumen fistula and the reticulo-omasal and omasal-abomasal orifices. In the first system of feeding, the milk is mixed with pregastric esterase prior to entering the abomasum. In the latter system, however, the milk by-passes

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the areas involved in pregastric esterase secretion. Six observations, three on each calf, were made with each feeding procedure. A minimum of three days was permitted to elapse between consecutive observations. The quantity of milk fed at each observation, ranging from 9.0 to 10.5 lb., was equal to 5% of the live weight of the calf. When the milk was fed orally, the rate of consumption was controlled, 1 lb/min, inasmuch as rate of consumption and length of the nursing period both have been shown to affect enzyme secretion (10). Rate of abomasal feeding was not regulated precisely, but this process usually required several minutes. At 1, 2, and 3 hr. after feeding, 150-200 ml. of abomasal contents were removed by suction through a rubber tube (8 mm. i.d.). The pH of each sample was measured immediately, after which it was placed in a water bath at 85° C. for 15 min. to inactivate the enzymes present. The sample then was cooled, homogenized in a Waring Blendor, and frozen for subsequent chemical analysis.

Analytical procedures. All samples of abomasal contents were analyzed for total free fatty acids according to the procedure of Keeney (4), as modified by Young *et al.* (14). In addition, the free fatty acids from the oral-fed samples were partitioned (9) into three fractions: butyric, valeric, and higher fatty acids. The level of free fatty acids in most of the abomasal-fed samples was too low to permit accurate evaluation of the different fractions.

RESULTS

When the milk was fed orally, the levels of total free fatty acids in abomasal contents (Table 1) were higher, particularly for Calf 1, than those observed when milk was administered abomasally. For Calf 1, the free fatty acids increased progressively in concentration during the sampling period, irrespective of method of feeding. For Calf 2, the level of free fatty acids remained fairly constant during this interval.

Changes in different free fatty acids that occurred in the presence of pregastric esterase (oral feeding) are presented in Table 2. The levels of butyric acid, similar for both calves, were highest in the 1-hr. samples and decreased gradually thereafter. In contrast, the levels of higher fatty acids were lowest in the initial samples and increased thereafter. Moreover, the two calves differed considerably in their abomasal concentrations of higher fatty acids. Though valeric acid is not a normal constituent of milk fat, the abomasal contents

TABLE 1

Effect of method of feeding on the mean concentration of total free fatty acids in abomasal contents

Method of feeding	Calf No. 1			Calf No. 2		
	Hours after feeding			Hours after feeding		
	1	2	3	1	2	3
	(μM of total free fatty acid per 5.0 g. of contents[a])					
Oral	100 ± 16	132 ± 6	162 ± 69	72 ± 19	71 ± 13	78 ± 11
Abomasal	10 ± 8	14 ± 15	23 ± 20	29 ± 11	25 ± 9	37 ± 16

[a] Mean of three observations ± standard error.

always contained a small quantity of this acid, usually highest in the 1-hr. samples.

Some discrepancy was noted between values obtained by the two analytical procedures used in this study. The figures obtained by summing the three fatty acid fractions (Table 2) averaged 6% higher than the values for total free fatty acids (Table 1) on corresponding samples of abomasal contents. Though the separation technique appeared to be more complete in its recovery of fatty acids, this difference was not statistically significant ($P > 10\%$). Much of the discrepancy probably was due to the difficulty encountered in obtaining representative aliquots from each sample for analysis. Even though the samples were homogenized in a Waring Blendor, the small particles of curd still tended to settle out.

The mean pH values of the abomasal contents for the oral and the abomasal methods of feeding, respectively, were as follows: 1 hr., 4.9, 4.6; 2 hr., 4.0, 4.0; and 3 hr., 3.3, 3.4.

Throughout this study, it was noted that the method of feeding affected the physical characteristics of the abomasal contents. When the milk was consumed

TABLE 2

Mean concentration of various free fatty acids in abomasal contents after feeding milk orally

Fatty acid fraction	Calf No. 1			Calf No. 2		
	Hours after feeding			Hours after feeding		
	1	2	3	1	2	3
	(μM of free fatty acid per 5.0 g. of contents[a])					
Butyric	36 ± 5	17 ± 5	8 ± 4	32 ± 11	22 ± 4	14 ± 3
Valeric	7 ± 4	4 ± 1.2	2 ± 1.6	7 ± 0.7	4 ± 1.2	4 ± 0.3
Higher	64 ± 18	102 ± 35	164 ± 79	42 ± 7	52 ± 15	75 ± 12

[a] Mean of three observations ± standard error.

orally, the curd particles were large, and the whey had a distinct watery appearance. In the abomasal-fed samples, the curd particles were small, and the liquid phase appeared milky, as if coagulation were incomplete. Conceivably, the foregoing change may have altered the rate of nutrient passage from the abomasum. Thus, an attempt was made to evaluate the effect of method of feeding on the rate at which one nutrient in milk, lactose, passes from the abomasum into the small intestine. Accordingly, the calves were fed on six additional occasions by each of the two procedures outlined previously. Samples of venous blood, for glucose determinations (7), were collected immediately before feeding and at 1, 2, 3, 4, 6, 9, and 12 hr. after feeding. Subsequent examination of these data indicated that the mean changes in blood glucose after each method of feeding were very similar and closely resembled those reported previously (6) for whole milk feeding. Thus, though evaluated indirectly, the manner in which the milk was administered did not appear to affect the rate of lactose passage from the abomasum.

DISCUSSION

Data presented in Table 1 indicate that pregastric esterase effects appreciable hydrolysis of milk fat in the abomasum. Furthermore, the marked reduction in total free fatty acids produced by minimizing the entrance of pregastric esterase into the abomasum suggests that this enzyme may be of greater significance than gastric lipase in this area of the alimentary tract. The extent to which the increase in acidity of abomasal contents reduces the activity of pregastric esterase is unknown. The optimum pH for the in vitro hydrolysis of milk fat by this enzyme is approximately 6.1, and 50% of maximum activity is present at pH 4.9 (13).

The relatively high specificity of pregastric esterase for glyceride linkages involving butyric acid (1-3, 9) was also confirmed in this study. Though milk fat averages only 10.4 *M* % of butyric acid (5), the level of this acid in the abomasum at 1 hr. post-feeding ranged from 28 to 43 *M* % of the total free fatty acids.

The decreasing level of butyric acid in abomasal contents between 1 and 3 hr. post-feeding, in contrast to an increasing concentration of higher fatty acids during the same interval, probably is related to several factors. The rate at which butyric acid is released initially by pregastric esterase is rapid, though it declines subsequently as the concentration of substrate is reduced. In addition, butyric acid probably passes from the abomasum at a relatively rapid rate because of its high solubility in water. The higher fatty acids, on the other hand, are released more slowly by pregastric esterase. Moreover, they are less soluble in water and probably remain entrapped within the curd particles to a considerable extent. Thus, the rate at which higher fatty acids leave the gastric area may be regulated primarily by the rate at which curd particles are reduced in size during abomasal digestion. The dissimilar behavior of the two calves with regard to changes in higher fatty acids (Table 2), though similar for butyric acid, may be related to the suggestion that pregastric esterase probably consists of at least two different esterases (9). If such enzymes possessed different substrate specificity patterns and were secreted in various ratios by different calves, variations in the fatty acid composition of abomasal contents would be expected.

The small particles of curd and the milky appearance of the whey associated with a minimum of pregastric esterase activity (abomasal feeding) suggest that this enzyme may be involved indirectly in the digestion of protein, though the absence of other salivary constituents may have produced the observed effect. In a previous study with calves, Wise *et al.* (11) reported that sham-fed whole milk, in which the fat had been hydrolyzed slightly by pregastric esterase, was coagulated by rennin in less time than whole milk not sham-fed. In contrast, the rennin coagulation time of sham-fed nonfat milk was similar to that of its unfed control. Though the sham-fed whole milk had a slightly lower pH than the unfed product, which may have hastened coagulation, it is possible that the presence of butyric acid or diglycerides accelerates the action of rennin also.

The origin of valeric acid in the abomasal samples is unknown. Conceivably,

it may have been produced by bacterial fermentation in the rumen, even though the calves were sustained entirely on milk. That this was the sole source seems unlikely, however, particularly in view of the fact that bands for acetic and propionic acids were never visible on the silicic acid columns during the separation procedure.

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MEASUREMENT OF THE NUTRITIVE VALUE OF ALFALFA AND TIMOTHY HAY BY VARIED TECHNIQUES [1]

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SUMMARY

Four mature rumen-fistulated Holstein cows were divided into two pairs and fed alfalfa and timothy hay by the double-reversal system over a 24-wk. period divided into four 6-wk. periods, in which the last 3 wk. of each period constituted the trial proper.

Digestibility of dry matter, protein, fiber, N-free extract, ether extract, energy, cellulose, lignin, and pentosans was determined by two procedures: (a) by the conventional procedure in which total feces were collected for ten-day periods, (b) by rumen technique, using Dacron bags containing weighed amounts of chopped forage which were placed in the rumen for periods of 48 hr. Content of the volatile fatty acids (VFA) in the rumen fluid was determined in samples taken on 38 occasions throughout the entire feeding period for each kind of hay.

Measured by the conventional procedure (total feces collection), timothy was more digestible than alfalfa in all entities except protein, N-free extract, and lignin. Measured by the rumen (Dacron bag) technique, timothy was on a par with, or more digestible than, alfalfa in all entities except protein and N-free extract.

Analysis of the rumen fluid showed content of VFA and ammonia to be higher when alfalfa or high-protein timothy was fed. Acetic acid constituted a slightly higher percentage of the total acidity when timothy was fed; this may have been responsible for a slightly higher butter fat test.

A recent report (1) of a 3-yr. study at this station of forage types for milk production showed that milk production (F.C.M.) was identical on alfalfa-grass mixed hay and mixed grass hay containing few or no legumes. More recent work (2), in which these hay types were supplemented with a 16% concentrate mix, showed that digestibility of fiber was uniformly higher in the ration containing the mixed grass type of hay, averaging 5.5% more than in the ration containing the alfalfa-grass type.

These results were contrary to common opinion as to the relative merits of alfalfa and the grasses for dairy cows. It was, therefore, decided to conduct further studies on the problem, using as nearly pure lots as possible of alfalfa and timothy hay. The objective was to determine, if possible, the fundamental nutritional reason(s) for the grasses producing as much milk as did the grass-legume mixtures. The investigation was divided into the following three phases: (a) Digestion trials by the conventional total feces collection procedure. (b) Digestion in the rumen as measured by the use of Dacron bags. (c) Determination of the pH of the rumen fluid and its content of ammonia and volatile fatty acids at a specific time on certain days.

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EXPERIMENTAL PROCEDURE

Five mature Holstein cows were rumen-fistulated during the summer of 1959; four of them were divided into pairs for use in the investigation, the fifth was held in reserve as a spare. The two pairs were fed alfalfa and timothy hay by the double-reversal system for four 6-wk. periods during the winter of 1959-60. The last 3 wk. of each 6-wk. period constituted the trials proper.

Reasonably pure stands of alfalfa were not available on the University farm, and except for one small lot which lasted only through the first 6-wk. trial the same was true of timothy. Second-cutting alfalfa (containing about 10% grass) was purchased from New York State through a reliable dealer, and early-cut (June 13) first-crop timothy from a local farmer. Hay constituted the sole ration, except for a salt–dicalcium phosphate mixture, and was fed at the rate of 2½ lb. per 100 lb. live weight.

Phase a. The digestion trials by the conventional total feces collection procedure were conducted during the last ten days of each 6-wk. period, immediately following the ten days devoted to Phase *b* (see below). In six of the 16 individual trials (four cows × four periods) individuals had to be removed after three to six days due to illness (pneumonia, lameness, inability to stand). This involved two of the four cows, one four times (No. 17), the other twice (No. 63). Separation of urine and feces for the first ten trials was by means of a harness described by Putnam *et al.* (9). This arrangement proved unsatisfactory, was responsible for most of the trouble experienced with Cow 17, and was finally abandoned in favor of inflatable catheters described by Cunningham *et al.* (6). These were used in the last six trials with complete satisfaction.

Feces were transferred to metal tubs several times daily, the total was weighed, and a 1% sample was taken with a special coring tube at the end of each 24-hr. period. The composite samples were refrigerated at 34° F. until the end of the ten-day period, at which time they were taken to the laboratory, where nitrogen was determined immediately by the Kjeldahl method in 10-g. quadruplicate charges of the fresh feces. The bulk of each sample was transferred to a shallow agateware pan and dried at 80° C. in a large air-convection thermostatically controlled oven, being stirred frequently to break the top crust. When thoroughly dry, usually in 8 hr., the samples were air-conditioned for several days, ground in a Wiley mill, conditioned again for about a week, and then bottled for analysis.

Fodder analyses of all materials (hay, orts, rumen residues, and feces) were determined by the usual A.O.A.C. procedure, sugar (in the hays only) by the method of Lane and Eynon (4), cellulose by the method of Druce and Wilcox (7), lignin by the method of Moon and Abou-raya as modified by Sullivan (12), pentosans by the official A.O.A.C. method (5), and gross energy in a Parr adiabatic calorimeter. Statistical analysis of the results was made according to the procedures of Snedecor (11).

Phase b. During the first ten days of each trial proper, four Dacron bags, each containing 30 g. of finely chopped hay (¾ in. or less), were placed in the rumen of each cow and allowed to remain for 48 hr., then removed, washed in

five changes of water, dried for 8 hr. at 100° C. in the above-mentioned oven, cooled in large desiccators, and weighed. This process was repeated twice during each ten-day period, so that data from 24 individual trials were available for each kind of hay for each 6-wk. period. There being four such periods during the course of the season, results were obtained from a total of 96 individual trials for each kind of hay.

Some detail as to placement and recovery of the bags may be of interest. Because bacterial action has been shown to be somewhat greater in the ventral than in the dorsal region of the rumen, an arrangement was devised that in theory, at least, would suspend the bags more or less vertically and assure that they would not float to the top of the rumen mass. This was done by placing the bags inside of slightly larger bags of the same material, both being closed by a drawstring and, as an extra precaution, by a stout elastic band. The outer bags had 2-in. loops at the top and bottom; the bottom loops were attached by means of split key rings to steel eyelets threaded into each end of a stainless steel cylindrical 2-lb. weight. Two bags were thus fastened to each end of the weight. The top loops of all four bags were gathered into a small harness snap, the distal end of which was attached to a length of 18-gauge nickel wire long enough to allow the steel weight to rest on the floor of the rumen. The wire was then passed through a small hole drilled in the cap of the rumen cannula and fastened around a small wooden splint with enough slack (2 or 3 in.) to allow for rumen motility. Figure 1 shows the assembly ready for insertion through the rumen cannula.

FIG. 1. Assembly for insertion through rumen cannula.

This procedure proved very satisfactory; only two bags in the entire series of 192 became detached from the train and they were readily recovered. On one occasion the spring snap and lead wire parted and it required a search of about half an hour to locate the bags. Other than this, no trouble was experienced; the cows offered no objection to the procedure and remarkably uniform results were obtained.

It was of interest to know how much of the loss in weight of the forage samples was due to bacterial action and how much was water-soluble material. This was determined by carrying through simultaneously with the rumen trials the same procedure with four bags suspended in a constant temperature water bath at 39° C., gently agitated by a small motor stirrer. To inhibit bacterial

action during the 48-hr. period in which these bags were in the water bath, they were immersed for a few seconds just prior to placement in the bath in a concentrated solution of mercuric chloride. Upon removal from the bath they were treated as described above for the bags that had been in the cows' rumens. The forage samples for this phase of the work were from the same portion of each lot of hay as those subjected to rumen digestion; all had been weighed out at one time and all were from the identical portion being fed to the cows at any given time. Samples were obtained by coring the lot of bales being fed at the time; these samples had been finely chopped in the coring process, maximum length being about ¾ in.

Phase c. Samples of rumen liquor were taken each time the Dacron bags were placed in or removed from the rumen, being obtained as a matter of convenience just before placement and after removal of the bags. The time of sampling was approximately 3 hr. after feeding. Taken at once to the laboratory, they were strained through four layers of cheese cloth and divided into two portions. One portion (100 ml.) was set aside for determination later of the concentrations of volatile fatty acids, being preserved with refrigeration and 2 ml. of saturated $HgCl_2$ solution. Volatile bases (as NH_3) were determined immediately in 100-ml. aliquots of the other portion by the method of Mach and Claus (8), as modified in this laboratory. Volatile fatty acids (acetic, propionic, butyric, and valeric) were determined by the column chromatographic procedure of Wiseman and Irvin (13), as modified by Stewart (13).

RESULTS AND DISCUSSION

Average composition of the hays appears in Table 1. The major differences of interest are the higher fiber, cellulose, and pentosan content, and the lower lignin content of the timothy hay. The relatively high protein content of the small lot of timothy hay from the University farm is a reflection of higher nitrogen supply.

TABLE 1

Average percentage composition of hays fed—winter of 1959-60

Kind of hay	Dry matter	Protein	Fiber	N-free extract	Ether extract	Ash	Sugar	Cellulose	Lignin	Pentosans
Alfalfa	88.8	16.6	32.0	44.4	1.9	5.2	6.1	19.4	9.8	16.0
Timothy (Univ. of Mass.)	88.9	16.9	29.6	41.5	2.7	9.4	4.4	22.3	6.7	19.2
Timothy (purchased)	88.1	11.7	35.7	43.3	2.9	6.4	4.1	26.6	6.6	22.2

A comparative summary of the results obtained by the total feces collection procedure (Phase *a*) and the Dacron bag technique (Phase *b*) is presented in Table 2. In general, the timothy was more digestible than the alfalfa, the differences in its favor being somewhat more marked by the conventional procedure than by the rumen technique. In both cases, six of the entities listed were more digestible in timothy than in alfalfa; in the former case (conventional pro-

cedure) all six differences were highly significant; in the latter, three were so.

The major contrasts between the two forages are in protein and N-free extract which favor the alfalfa and in fiber, cellulose, and pentosans which favor the timothy. The difference in fiber digestibility is worthy of special mention, being over 28% higher in the timothy by the conventional procedure and over 23% higher by the rumen technique. This highly significant difference confirms, and is even more striking than, a similar finding reported last year (2), referred to in the introduction to this paper. It has been stated in a more recent paper (3) that "the reason for the much higher digestibility of both fiber and cellulose in the timothy hay is due, in our opinion, to its considerably lower average lignin content" (6.6% *cf.* 9.8%). A glance at the values for lignin by the conventional procedure in Table 2 would seem to refute this statement, the lignin in the alfalfa being nearly 4% more digestible than the lignin in the timothy. However, the high standard errors here (4.0 and 3.1) render this difference nonsignificant, and it is a matter of general knowledge that digestion coefficients for lignin are apt to have little meaning. Curiously enough, a very different result for digestibility of lignin is noted in the values obtained by the rumen (Dacron bag) technique.

There are several interesting differences between the two methods of evaluating the forages; to save space, the conventional procedure will be referred to below as *a*, the rumen (Dacron bag) technique as *b*. The differences worthy of note follow in descending order of their appearance in Table 2.

TABLE 2

Summary of differences in digestibility between alfalfa and timothy hay (Winter of 1959-60)

Entity	Total feces collection procedure			Dacron bag technique		
	Alfalfa	Timothy	Difference	Alfalfa	Timothy	Difference
Dry matter	58.9 ± 0.4	66.9 ± 0.8	8.0**	66.4 ± 0.7	66.5 ± 0.8	0.1
Protein	72.0 ± 1.3	60.8 ± 1.4	11.2**	88.1 ± 0.6	80.1 ± 0.9	8.9**
Fiber	40.0 ± 0.5	68.2 ± 1.3	28.2**	33.3 ± 3.2	56.0 ± 1.4	22.7**
N-free extract	72.1 ± 4.0	67.2 ± 0.7	4.9**	79.4 ± 0.4	66.4 ± 1.1	13.0**
Ether extract	17.9 ± 2.9	46.1 ± 1.4	28.2**	73.5 ± 2.0	75.6 ± 1.5	2.1
Energy	56.9 ± 0.5	63.9 ± 0.8	7.0**	64.7 ± 0.6	65.0 ± 1.0	0.3
Cellulose	48.8 ± 0.5	70.2 ± 1.3	21.4**	42.3 ± 1.6	57.7 ± 1.8	15.4**
Lignin	13.5 ± 4.0	9.8 ± 3.1	3.7	19.9 ± 0.9	29.3 ± 2.5	9.4**
Pentosans	54.2 ± 0.6	68.2 ± 0.6	14.0**	50.0 ± 0.9	56.5 ± 1.4	6.5**

Wherever the differences are indicated as highly significant, $P < .005$ in all cases, as low as .001 in some.

1. The somewhat higher digestibility of the protein of both forages by *b* and than by *a*. Presumably, this is due to the presence of metabolic nitrogen in the feces.

2. The consistently lower values by *b* for the structural carbohydrates, viz: fiber, cellulose, and pentosans. This is considered to be due to the fact that the forage enclosed in the Dacron bags did not have the benefit of rumination, which would materially reduce particle size and thus facilitate bacterial breakdown

of these structural materials. This situation is recognized as a defect in Procedure *b*.

3. The much higher values for ether extract by *b*. This is considered to be due to the formation of insoluble calcium soaps in the intestine, which appear as ether extract in the feces and result in low values by *a*. Dicalcium phosphate was fed daily and would be an important source of calcium for this reaction; also, the relatively high calcium content of alfalfa could be responsible for ether extract in that forage to nearly 30% less than that noted for timothy.

Table 3 (a) and (b) reveals for the most part a remarkable uniformity between individuals in the digestion coefficients for the individual forages of most of the entities determined, regardless of procedure used. The exceptions to this situation were the values for ether extract and lignin. Values for alfalfa between individuals tended to be slightly more uniform than those for timothy. Especially worthy of note for their uniformity are the values for energy, fiber, cellulose, and pentosans.

TABLE 3

Summary of digestion coefficients obtained with the individual cows (Winter of 1959-60)

(a) Conventional (total feces collection) procedure [a]

Entity	Cow 8		Cow 17		Cow 62		Cow 63	
	Alfalfa	Timothy	Alfalfa	Timothy	Alfalfa	Timothy	Alfalfa	Timothy
Dry matter	59.6	67.3	59.1	68.3	58.5	66.3	58.5	65.9
Protein	70.6	60.7	71.9	56.8	73.8	59.2	71.7	64.0
Fiber	41.2	69.8	39.4	69.5	40.5	68.9	38.9	65.2
N-free extract	72.6	66.6	72.5	70.2	70.8	66.9	72.4	66.1
Ether extract	13.6	47.4	15.7	46.2	23.7	46.0	18.5	43.5
Energy	57.7	64.3	57.4	65.2	56.4	63.8	56.0	62.2
Cellulose	50.0	70.2	47.1	71.9	48.9	71.6	48.8	71.8
Lignin	Not averaged—values ranged from 0 to 26.4							
Pentosans	55.9	68.2	54.6	69.3	53.3	68.1	52.8	67.6

(b) Rumen technique (using Dacron bags) [b]

Dry matter	66.0	65.2	66.0	67.1	68.3	68.9	65.4	65.2
Protein	86.7	77.5	87.4	76.2	89.6	82.0	88.6	80.8
Fiber	32.0	56.3	33.0	52.5	37.0	58.0	31.5	52.7
N-free extract	79.4	64.8	78.7	68.4	80.6	68.8	78.8	64.7
Ether extract	73.6	71.7	67.1	76.0	80.5	79.9	72.8	74.8
Energy	64.3	63.2	64.2	66.7	66.6	67.5	63.8	62.3
Cellulose	40.6	57.4	40.6	58.3	47.6	60.7	40.6	54.9
Lignin	22.3	33.2	21.3	31.1	18.8	28.2	17.3	33.5
Pentosans	48.4	56.3	49.8	59.4	52.3	58.2	49.4	56.6

[a] Each value reported above is the average of two individual trials.
[b] Each value reported above is the average of eight individual trials.

Coming to consideration of results from analysis of the rumen fluids, these are available from a total of 76 samples and are reported in detail in Table 4 (a) and (b):

1. All entities determined were higher when alfalfa was fed, all differences except that for acetic acid being highly significant.

2. Ammonia nitrogen was over twice as high with alfalfa as with timothy.

TABLE 4a

Content of volatile fatty acids [a] and ammonia [b] in the rumen fluid

Cow No.	Kind of hay	Acetic Acid	Propionic Acid	Butyric Acid	Valeric Acid	Total acids	Ammonia	pH of rumen fluid
8	Alfalfa	8.95 ± .30	2.45 ± .13	1.56 ± .09	0.52 ± .02	13.48 ± .54	19.11 ± 1.11	6.50 ± .05
	Timothy	8.72 ± .29	2.05 ± .05	1.31 ± .04	0.42 ± .01	12.50 ± .39	6.88 ± .91	6.10 ± .04
17	Alfalfa	8.93 ± .42	2.44 ± .11	1.60 ± .06	0.51 ± .02	13.48 ± .61	17.01 ± .74	6.34 ± .08
	Timothy	8.90 ± .34	2.28 ± .10	1.37 ± .04	0.47 ± .01	13.02 ± .49	7.53 ± .72	6.02 ± .07
62	Alfalfa	9.80 ± .28	2.73 ± .09	1.41 ± .05	0.64 ± .02	14.58 ± .44	18.77 ± 1.41	6.44 ± .04
	Timothy	8.92 ± .44	2.07 ± .11	1.24 ± .04	0.41 ± .02	12.64 ± .61	10.41 ± 1.69	6.08 ± .05
63	Alfalfa	9.20 ± .23	2.59 ± .13	1.47 ± .05	0.60 ± .01	13.86 ± .42	19.69 ± 1.50	6.50 ± .03
	Timothy	8.55 ± .39	2.18 ± .08	1.26 ± .05	0.39 ± .02	12.38 ± .54	11.06 ± 1.41	6.07 ± .10
Average	Alfalfa [c]	9.28 ± .22	2.54 ± .06	1.51 ± .04	0.57 ± .01	13.90 ± .33	18.69 ± .76	6.45 ± .03
	Timothy [c]	8.80 ± .20	2.15 ± .05	1.29 ± .02	0.42 ± .01	12.66 ± .28	8.88 ± .92	6.07 ± .01

[a] Expressed as millimoles of VFA per 100 ml. of rumen fluid.

[b] Expressed as milligrams of ammonia nitrogen per 100 ml. of rumen fluid.

[c] Each of these values is the average of 38 individual samples. With the exception of the values for acetic acid, all the average differences between the hays are significant at or beyond the 0.1% level ($P < 0.01$). The difference in acetic acid is not significant.

TABLE 4b

Content of the several acids expressed as a percentage of the total acidity

Cow No.	Kind of hay	Acetic Acid	Propionic Acid	Butyric Acid	Valeric Acid	Total
8	Alfalfa	66.3	18.2	11.6	3.9	100.0
	Timothy	69.7	16.4	10.5	3.4	100.0
17	Alfalfa	66.2	18.1	11.9	3.8	100.0
	Timothy	68.4	17.5	10.5	3.6	100.0
62	Alfalfa	67.2	18.7	9.7	4.4	100.0
	Timothy	70.6	16.4	9.8	3.2	100.0
63	Alfalfa	66.4	18.7	10.6	4.3	100.0
	Timothy	69.0	17.6	10.2	3.2	100.0
Average	Alfalfa	66.7	18.3	10.9	4.1	100.0
	Timothy	69.5	17.0	10.2	3.3	100.0

If we exclude the ammonia values obtained for Cows 62 and 63 when fed the high-protein timothy in Period 1 [2] which Cows 8 and 17 did not receive, the difference is even more marked; the ammonia nitrogen from alfalfa is over threefold that from timothy. One would expect that with the somewhat higher protein (or nitrogen) level in alfalfa more ammonia would be formed than with the timothy, but a threefold amount is out of proportion to their respective protein levels (16.6% *cf* 11.7%). Some factors other than protein intake are involved here; presumably, one factor is more rapid breakdown of the alfalfa proteins to their constituent amino acids with concomitant rapid deaminization of the latter. Two questions at once arise: Is this relatively high concentration of ammonia in the rumen desirable? Might it be part of the explanation for the fact that in our work over a period of years as much milk has been produced on grass hays as on grass-legume hays?

It should be noted that when the lot of timothy from the University farm which contained 16.9% of protein was fed to Cows 62 and 63, the ammonia nitrogen averaged 16.5 mg. %, in comparison with alfalfa at 19.2 mg. %. This situation accounts for the somewhat higher average values for these two cows (10.4 and 11.1) than for Cows 8 and 17 (6.9 and 7.5), which did not receive this high-protein timothy. In these studies, at least, high protein content, regardless of forage type, resulted in relatively high levels of ammonia in the rumen fluid.

It is emphasized that all of these values for the several entities determined represent only the concentration in the rumen fluid at a specific time and are not a measure of total production. No one has yet devised a reliable means of measuring actual production of volatile fatty acids or any other entity in the rumen. This would be extremely difficult to accomplish, and in an open system such as exists there the validity of any such measurement would be open to question.

3. In view of the higher ammonia values when alfalfa was fed, it is not sur-

[2] See Table 1 and lines 2 and 3, page 2235.

prising that the pH of the rumen fluid was considerably higher. Conversion of the average pH values to their equivalent H-ion concentration shows this latter value to be not much more than half as high in the alfalfa as in the timothy.

4. The remarkable uniformity of results between cows of individual acid values and for total acid content is worthy of more than passing mention. The range between maximum and minimum values when alfalfa was fed was 0.86% for acetic acid, 0.29% for propionic acid, 0.23% for butyric acid, and 0.19% for valeric acid. When timothy was fed, the range was somewhat less, 0.37% for acetic, 0.23% for propionic, 0.13% for butyric, and 0.08% for valeric acid. Considering the complexity of such a system as exists in the rumen, and the many variables which can affect its continuous processing of feed, a considerable degree of confidence can be placed in these results.

5. A similar degree of uniformity is noted for ammonia content when alfalfa was fed. A reason has already been given for the lack of uniformity in ammonia content between the two pairs of cows when timothy was fed.

In Table 4b the content of the several acids has been converted to percentages of the total acidity. The point of interest here is the consistently higher percentage of acetic acid in the rumen fluid when timothy was fed, the percentages of the other three acids being correspondingly lower. The much higher digestibility of fiber in the timothy (see Table 2) may account for the higher concentration of acetic acid. Shaw (10) has shown that high-roughage rations, i.e., rations high in fiber, result in greater production of acetic acid relative to propionic acid, and vice versa. Since acetic acid is an important source of the short-chain fatty acids in milk, it is not surprising to note from Table 5 a slight, but not significant, difference in fat test in favor of the timothy hay.

TABLE 5

Fat percentages in milk

Ration	Cow 8	Cow 17	Ration	Cow 62	Cow 63	Sampling dates [a]
Alfalfa	3.9	2.6	Timothy	3.3	3.3	Nov. 23-27
Timothy	3.8	3.1	Alfalfa	3.2	3.3	Jan. 4-8
Alfalfa	4.1	3.1	Timothy	3.6	3.3	Feb. 15-19
Timothy	4.1	3.1	Alfalfa	3.7	3.1	Mar. 28-April 1

Average—on alfalfa—3.375
—on timothy—3.450.

Average change when shifted—from alfalfa to timothy—+.133
—from timothy to alfalfa—+.017.

[a] Five-day composite samples in all cases.

Comparison of the values obtained by the rumen technique with the water bath controls showed an average of about 25% water-soluble dry matter in alfalfa and about 20% in timothy. This means that bacterial action was responsible for about 41% of the processed dry matter in the alfalfa, and for about 45% in the timothy.

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EFFECT OF PARTICLE SIZE UPON THE IN VITRO CELLULOSE DIGESTIBILITY OF FORAGES BY RUMEN BACTERIA[1, 2, 3]

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SUMMARY

Rates of in vitro cellulose digestion were determined for mature timothy (seed ripe) which had been ball-milled 0, 6, 24, and 72 hr. The total amount of cellulose digested increased with the time of ball-milling up to 72 hr. Based on this observation four stages of timothy, three of alfalfa, and two each of brome and orchard grass, along with 72-hr. ball-milled samples of these same forages, were used as substrates for in vitro rumen fermentations. In all cases, ball-milling increased the amount of cellulose digested, and this increase became larger with advancing maturity and lignification of the forage. In further work, mature timothy was digested 48 hr. in vitro and the residue isolated. Less than 5% of the cellulose remaining in this residue was digested in a second 48-hr. fermentation; however, when a portion of this residue was ball-milled, 67% of the cellulose could be digested. Additional experiments which determined the amount of forage cellulose soluble in cupriethylene diamine, indicated that the amount of cellulose which can be dissolved decreases with forage maturity and increased deposition of lignin. The amount of cellulose dissolved was markedly increased after ball-milling, and again this increase became larger with advancing maturity of the forage. The experimental evidence presented in this paper substantiates the theory that the lignin in forages acts as a physical barrier between the cellulose and cellulolytic rumen bacteria.

Microscopic examination of cellulose fibers during digestion suggested to Baker and Harriss (1) that the lignin in forages is a physical barrier between the cellulose and cellulolytic rumen bacteria. Using plant histology methods, Drapala *et al.* (8) determined the degree of lignification of red clover at five different periods of maturity, and related this information to cell digestibility as observed microscopically in fecal residues. In general, they found an increase in the number and size of undigested bits of plant tissues with an increase in maturity of the forage eaten and its increasing lignification. From this, they tentatively concluded that only lignified parts of the plant appear in quantity in the feces, excepting substances encased by a lignified cell wall.

In more recent work, Kamstra *et al.* (10) measured the in vitro digestibility of cellulose in forages, along with the digestibility of cellulose and holocellulose isolated from the same forage. They found that the maturity of plant material has an effect upon cellulose digestion. As the plant matures and increases in lignin content, the amount of cellulose which can be digested decreases. How-

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ever, the effect of maturity diminished greatly when the cellulose was chemically separated from the plant material, either as cellulose or holocellulose. These authors also found that isolated lignin had no effect on purified cellulose digestibility when it was added to an in vitro rumen fermentation. Quicke and Bentley (15) tried to correlate in vitro cellulose digestion and lignin content with four stages of timothy and two stages each of brome and orchard grass. The digestibility of the four stages of timothy appeared to be related to their acid-insoluble lignin content; however, they concluded that this relationship did not exist with the brome and orchard grass samples. As a result of this and other studies reported in the literature (12, 14), the theory has been proposed that the extent of deposition of lignin around the cellulose fiber (forming a physical barrier), rather than the total concentration of lignin, is probably responsible for the decreased digestibility of forages as they mature.

Additional support for this proposal is found in a report by Baker *et al.* (2), who found that the rate of digestion of purified cellulose was inversely proportional to its crystallinity. When hays or the cellulose and holocellulose isolated from these hays were subjected to X-ray diffraction, the cellulose was found to be primarily in the amorphous state. Similar results on plant celluloses were obtained by Tomlin and Davis (16). Since amorphous cellulose is readily attacked by rumen cellulolytic bacteria, these authors concluded that if physical contact can be made between a forage plant cellulose in its native state and the microorganisms or microbial cellulases, the cellulose should be digestible.

If such a protective lignin sheath exists, then disintegration of the forage particle could increase the amount of cellulose available for digestion through physical rupture of this barrier. Several reports have appeared which touched on this subject; however, the results were rather contradictory. Virtanen (17) studied the cellulose digestibility of birch, pine, and aspen dusts with cellulolytic rumen bacteria. The finer the wood was ground, the more cellulose was fermented. On the other hand, Lawton *et al.* (11) reduced the particle size of basswood to the order of cellular dimensions without an increase in the amount of cellulose which could be digested by rumen bacteria.

In a previous report from this laboratory (5), it was shown that the particle size of purified cellulose could be markedly reduced by ball-milling, and that this reduced particle size resulted in a shorter lag-phase for digestion by rumen bacteria in vitro, with no marked difference in the rate or extent of digestion. The results reported herein deal with the in vitro cellulose digestibility of forages after the forage particle size has been reduced by grinding in a ball-mill.

EXPERIMENTAL PROCEDURE

Samples of rumen ingesta were obtained, through a permanent rumen fistula, from a steer maintained on a diet of mixed hay. The fluid portion of the ingesta was expelled, and a suspension of rumen bacteria was obtained by washing the pressed rumen ingesta with phosphate buffer as described by Johnson *et al.* (9). The 250 × G supernatant of this suspension was used as an inoculum (7). The basal media and general in vitro procedure have been described previously

(5). Fermentations were carried out in 250-ml. flasks, calibrated between volumes of 100 and 175 ml., and time samples were taken at 6, 12, 24, 30, and 48 hr. (5). Three and one-half grams of forage substrate and an amount of inoculum equivalent to 44 ml. of the whole bacterial suspension were used.

The forages used in this investigation are described in Table 1. Samples

TABLE 1
Description of forages used and their lignin contents

Forage	Date cut	Description	Lignin (%)
Timothy, 1st stage	5-24-57	Vegetative stage	8.5
Timothy, 2nd stage	6- 7-57	Heading	11.9
Timothy, 3rd stage	6-20-57	Headed	15.3
Timothy, 4th stage	7-16-57	Seed ripe	17.0
Bromegrass, 1st stage	6-10-57	Early bloom	17.7
Bromegrass, 2nd stage	6-26-57	Seed stage	19.0
Orchardgrass, 1st stage	6- 6-57	Early bloom	14.8
Orchardgrass, 2nd stage	6-18-57	Early seed stage	17.4
Alfalfa, 1st stage	5-18-56	Early bloom	8.0
Alfalfa, 2nd stage	6-26-56	Late bloom	11.8
Alfalfa, 3rd stage	7-17-56	Seed stage	13.8
Red clover	9-29-60	Full bloom	12.9
Bird's-foot-trefoil	9-29-60	Pre-bloom	11.7

were cut by hand, dried either in air or a forced-draft oven at 65° C., and ground in a Wiley mill through a 40-mesh screen.

Four per cent suspensions of each forage, in water, were ball-milled in a porcelain jar with stones as a charge. The 40-mesh forages, ball-milled forage suspensions, and time samples obtained after fermentation were analyzed for cellulose by the procedure of Crampton and Maynard (4). Retention of the fine particles obtained by ball-milling in the asbestos pad used in the Crampton-Maynard cellulose analysis, as well as recovery of cellulose added to the ball mill, were found to be satisfactory in the previous study on purified cellulose (5). Lignin content of the forages was estimated according to the method of Patton (13).

Solubility of forage cellulose in cupriethylene diamine was determined according to a method developed in this laboratory, the main details of which are reported in Abstract form by Dehority and Johnson (6).

RESULTS AND DISCUSSION

The purpose of the first experiment undertaken in this study was to determine whether the amount of cellulose digested in vitro could be increased in a very mature forage by ball-milling, and if so, what time of ball-milling would be optimum. Samples of fourth-stage timothy, ball-milled 0, 6, 24, and 72 hr., were used as substrates for a series of in vitro rumen fermentations, and the results are presented in Figure 1. Four separate fermentations were run, using all four substrates in each, and time samples were taken at 6, 12, 24, 30, and 48 hr. As can be seen, there was a reduction in lag-phase after ball-milling similar to that found with purified cellulose (5). However, the most impor-

tant observation was considered to be that the amount of cellulose digested was increased in direct relationship with the length of ball-milling. In an additional experiment, ball-milling for 96 hr. did not result in any further increase in cellulose digestion. Since the digestion of each of the substrates plateaued after approximately 30 hr., and the height of the plateau increased with length of ball-milling, these data would suggest that forage cellulose was readily digestible and that the amount digested is governed by its availability to the cellulolytic rumen bacteria.

Using the 72-hr. period, the effect of ball-milling upon digestibility was studied in several different forages at various stages of maturity. Figure 2

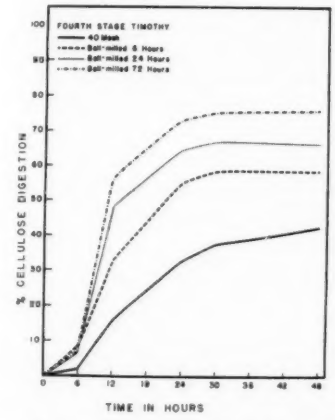

FIG. 1. Effect of time of ball-milling upon the in vitro cellulose digestion of mature timothy.

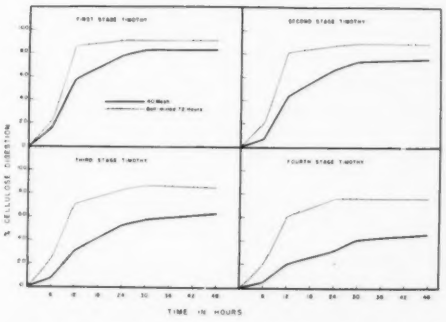

FIG. 2. Time curves of cellulose digestion by rumen bacteria, using four stages of timothy ball-milled 0 and 72 hr. as substrates. Fermentation sampled at 6, 12, 24, 30, and 48 hr.

shows the rate of cellulose digestion obtained for the four stages of timothy. Time samples were taken at 6, 12, 24, 30, and 48 hr. and four fermentations were run with each substrate. In all cases, the 40-mesh and ball-milled sample for a particular stage were run simultaneously. It is of particular interest that ball-milling increased the total cellulose digestibility, and the amount of increase became larger as the timothy matured. The 48-hr. cellulose digestibility values for the 40-mesh samples were 83.0, 75.7, 62.4, and 45.6 for Stages 1-4, respectively, whereas digestibilities after ball-milling were 91.0, 88.5, 85.4, and 75.8.

Two stages each of bromegrass and orchardgrass (described in Table 1) were subjected to ball-milling; however, only the total digestibility in a 48-hr. fermentation was determined. These data are presented in Table 2, and show a response similar to that obtained with timothy.

Because of the results obtained with the grass hays, it was believed that an investigation of legumes might be of interest. Figure 3 presents the rate of cellulose digestion for three stages of alfalfa. As with the grasses, an increase in cellulose digestibility was obtained after ball-milling. One sample each of

TABLE 2

Effect of ball-milling upon the 48-hr. in vitro cellulose digestibility of bromegrass and orchardgrass [a]

Forage	Cellulose digested (%)	
	Ground (40-mesh screen)	Ball-milled (72 hr.)
Bromegrass, 1st stage	53.1 ± 3.6 [b]	80.5 ± 1.2
Bromegrass, 2nd stage	42.7 ± 3.2	75.8 ± 1.3
Orchardgrass, 1st stage	64.6 ± 4.8	83.1 ± 3.0
Orchardgrass, 2nd stage	53.7 ± 2.2	82.3 ± 1.4

[a] Five fermentations were carried out with each sample of bromegrass, and three with each sample of orchardgrass.

[b] Mean and standard deviation.

red clover (full-bloom) and bird's-foot-trefoil (pre-bloom) were also tested. The cellulose digestibilities obtained before and after ball-milling were 55.6 and 75.0% for red clover and 62.5 and 77.0% for bird's-foot-trefoil, which is in line with previous data.

Examination of the lignin content and total cellulose digestibility of the grasses and legumes revealed some rather marked differences, Table 3. The data

TABLE 3

Lignin content and cellulose digestibility of several grasses and legumes

Forage	Lignin (%)	Cellulose digested (%)
Timothy, 1st stage	8.5	83.0 ± 2.8 [a]
Alfalfa, 1st stage	8.0	67.8 ± 2.5
Timothy, 2nd stage	11.9	75.7 ± 1.9
Alfalfa, 2nd stage	11.8	58.1 ± 2.7
Bird's-foot-trefoil	11.7	62.5 ± 1.7
Timothy, 3rd stage	15.3	62.4 ± 0.8
Alfalfa, 3rd stage	13.8	54.8 ± 1.5
Red clover	12.9	55.6 ± 1.3
Orchardgrass, 1st stage	14.8	64.6 ± 4.8

[a] Mean and standard deviation.

are divided into three groups, in which both the legumes and grasses have approximately the same lignin content. At about the same lignin content, the legume cellulose was digested to a lesser extent than the cellulose in the grass. From these data one might conclude that some basic difference existed between the grasses and legumes with regard to the amount of cellulose digested per given amount of lignin.

As can be observed in Figures 1, 2, and 3, cellulose digestion had plateaued after 30-hr. fermentation, with no marked increases occurring up to 48 hr. However, the question arose as to what effect prolonged fementation might have had on the cellulose available for digestion. To evaluate this point, a large scale in vitro fermentation was conducted in which fourth-stage timothy was fermented for 48 hr., during which 44.5% of the cellulose was digested. The residual material was then separated by centrifugation, washed, dried, and

reground in a Wiley mill through a 40-mesh screen. A portion of this material was then ball-milled, and both the 40-mesh and ball-milled samples were used as substrates for a second in vitro fermentation. The rates of cellulose digestion for these samples are given in Figure 4. No appreciable amount of the cellulose

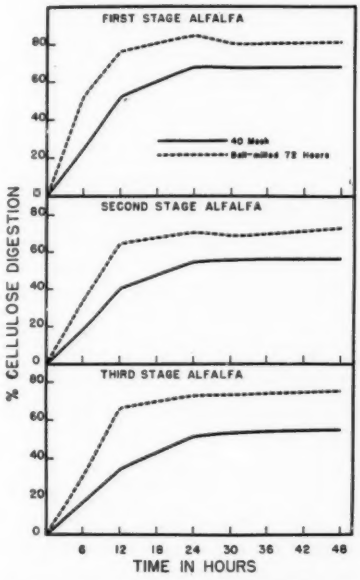

FIG. 3. Time curves of cellulose digestion by rumen bacteria, using three stages of alfalfa ball-milled 0 and 72 hr. as substrates. Fermentation sampled at 6, 12, 24, 30, and 48 hr.

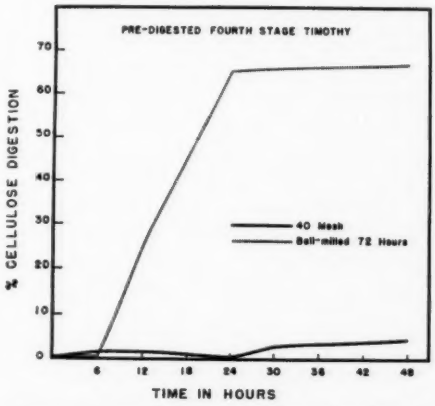

FIG. 4. Time curves of cellulose digestion by rumen bacteria, using predigested mature timothy ball-milled 0 and 72 hr. as substrates (see text). Fermentation sampled at 6, 12, 24, 30, and 48 hr.

in the residue was digested in this second fermentation; however, ball-milling of the residue allowed about 67% of the remaining cellulose to be digested.

These data support the supposition that some kind of physical barrier exists between the cellulose and the cellulolytic rumen bacteria. Ball-milling is a physical treatment and apparently does not change the chemical structure of any of the constituents in the forage. Thus, the lignin and other components are still present in the fermentation mixture; only their physical relationship has been disrupted. The evidence presented for the theory of a physical barrier existing in forages is rather indicative of what the situation might be; however, it is still circumstantial. Thus, an additional experiment was devised to give more insight into this problem. Table 4 shows the amount of cellulose which could be dissolved from the different stages of timothy (40-mesh and ball-milled) by cupriethylene diamine, a cellulose solvent. The amount of cellulose which could be dissolved from the 40-mesh timothy samples decreased with increasing maturity of the plant. After ball-milling, the per cent of cellulose which could

TABLE 4
Per cent forage cellulose dissolved by cupriethylene diamine

Forage	Cellulose dissolved (%)	
	Ground (40-mesh screen)	Ball-milled (72 hr.)
Timothy, 1st stage	78.2 ± 2.2[a]	86.0 ± 1.5
Timothy, 2nd stage	72.9 ± 1.6	87.2 ± 1.0
Timothy, 3rd stage	61.7 ± 1.7	82.8 ± 1.6
Timothy, 4th stage	49.7 ± 1.9	79.4 ± 0.8

[a] Mean and standard deviation.

be dissolved was increased, the amount of increase becoming larger with the maturity of the forage. In general, the data obtained on cellulose solubility were very similar to those obtained on in vitro cellulose digestibility. Similar increases were found in the amount of cellulose dissolved after ball-milling samples of brome and orchardgrass.

The data presented in this paper would support the following conclusions: (1) The amount of forage cellulose digested by rumen bacteria decreased with increasing maturity and lignification of the plant; (2) the total amount of cellulose in a forage available for digestion by rumen bacteria was almost completely utilized after 30 hr. of fermentation in vitro; (3) physical reduction of the forage particle size by ball-milling increased the amount of cellulose which could be digested in vitro and this increase was directly related to the length of ball-milling, at least up to 72 hr.; (4) the increase in in vitro cellulose digestibility due to ball-milling became larger as the forage matured and lignin deposition was increased; (5) there appeared to be a basic difference in grasses and legumes in regard to the amount of cellulose which could be digested per given amount of lignin in the plant; and (6) simple physical solubility of the forage cellulose in cupriethylene diamine decreased with maturity, while the cellulose solubility was markedly increased after ball-milling. These conclusions support the theory that the decreased cellulose digestibility of mature forages is caused by a physical barrier of lignin between the bacteria and the cellulose, and not the total concentration of lignin. Perhaps the description given by Clarke (3), where he considers the cell wall as a continuous interpenetrating system of cellulose and lignin similar to reinforced concrete, in which the iron rods represent the cellulose frame work and the concrete the lignin and other constituents, would best fit the actual spatial relationship between cellulose and lignin in the mature plant.

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EFFECTS OF DIETARY SODIUM FLUORIDE ON DAIRY COWS. VI. IN YOUNG HEIFERS[1]

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SUMMARY

The effects of increasing levels of sodium fluoride to ascertain the tolerance of the dairy cow and the influence of initial age, restricted feed intake, time interval, and prior exposure of the dam on subsequent fluorine ingestion of the offspring, have been studied.

On the basis of fluorine retention in bones, urine excretion, weight gain and adult weight, systemic physiological reactions, tooth scores, molar wear, and general health and condition, the tolerance of young dairy cattle to fluoride was less than 1.6 mg. F/kg of body weight daily. Cows receiving 1.4 mg. F/kg were in a marginal zone of toxicity. The toxicity was unaffected by a variation of initial age from 6 wk. to 6 mo. or by prior exposure of the dam to fluorine ingestion. The restriction of grain intake resulted in a slight depression of adult body weight independent of fluoride level.

The data indicated that one of the most reliable measures of fluorine toxicity was the presence of skeletal fluoride levels in excess of 5,500 p.p.m.

This study is the third in a series of experiments designed to follow the development of fluorine toxicosis in dairy cows under controlled conditions. The first of these, Experiment 809A, utilized 2-yr.-old bred heifers (11, 12, 15, 16), whereas the second, Experiment 809B, was concerned with the results of feeding graded levels of sodium fluoride to mature cows, beginning at approximately 5 yr. of age (13). These experiments demonstrated that 50 p.p.m. of fluoride in the diet, resulting in the ingestion of from 1.7 to 1.8 mg/kg of body weight per day during heavy lactation and a yearly average of 1.5 mg. of fluorine/kg of body weight, produced some evidence of fluorosis in two cows out of three by the third year of exposure. Mature cows under the same conditions were able to tolerate 50 p.p.m. for 3 yr. and through three lactation periods without any physiologic evidence of fluorosis. The general problem of fluorine toxicity in cattle is discussed in recent reports and reviews (4, 5, 7, 8, 10).

It has been shown (7, 14) that young, actively growing laboratory animals deposit more fluorine in their developing skeleton than do older animals of the same species. The initial age of exposure to elevated levels of fluorine may,

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therefore, provide an important variable in determining the degree of fluorosis in domestic animals.

The study herein reported was set up to determine what effect the initial age at first exposure had upon the course of the development of fluorine toxicosis. The following data were, therefore, sought: (a) effect of initial age, ranging from 6 wk. to 6 mo., upon the time interval required to develop fluorosis in young dairy cattle; (b) fluorine tolerance level; (c) effect of reduced grain intake on development of fluorosis; and (d) effect of fluorine ingestion by the dam upon subsequent development of fluorosis in the offspring.

EXPERIMENTAL PROCEDURE

Twenty-eight Holstein calves from 6 to 27 wk. of age were divided into six lots, a control without added fluorine, and five experimental lots. The animals in Lots 2-6 were given NaF to supply 1.0, 1.2, 1.4, 1.6, and 2.0 mg. of F/kg of body/weight/day, respectively. Within each lot some of the calves were selected from dams previously fed fluoride experimentally (12, 13) and the remainder were obtained from grade herds in the vicinity of Madison. Two calves in Lot I (control), and three calves in Lots III and IV fed 1.2 and 1.4 mg. of fluorine per kilogram of body weight were held to a reduced grain intake. The detailed experimental design is presented in Table 1. The cattle were fed legume-grass silage, mixed hay, and 2 lb. of grain a day prior to calving. Thereafter, the grain mixture (12) was fed at the rate of 1 lb. of grain per 4 lb. milk produced. Animals on the reduced grain intake received only roughage from 6 mo. of age to calving and half the normal grain ration thereafter.

The heifers were not allowed on pasture but were turned out daily for exercise and observation of signs of estrus. They were bred artificially. Reagent grade sodium fluoride was mixed into the grain mix to give a concentration of 8 mg. of fluorine per gram and the correct portion of this was weighed and placed on the top of the grain ration, twice daily. The cattle were fed the grain and silage in individual boxes to assure correct dosage. The amount of fluorine administered was adjusted every 2 wk. when the animals were weighed. Fluorine analyses were obtained by the Alcoa Research Laboratory modification (1) of the Willard and Winter method (18). The majority of the cattle were removed from the experiment either during or at the end of the second lactation, with the following exceptions: Cow No. 3, Lot 1; Cows No. 9 and 10, Lot 3; Cows No. 12 and 14, Lot 4; and Cows No. 22 and 23, Lot 6. One cow from each pair was fed the control diet (no fluoride added), whereas the other was continued on the fluorine-supplemented diet.

Daily milk production was recorded and milk fat calculated from the fat percentage determined by the local DHIA testing service. When permanent teeth began to erupt, frequent oral inspections were made and the teeth scored by the method of Hobbs *et al.* (3). Urine samples were collected every few months and tail vertebrae biopsies were performed after 18 and 30 mo. of exposure. When the experiments were terminated, the following bones were taken for study and analyses: metatarsus, metacarpus (as examples of compact

TABLE 1

Information on animals used in Experiment 809C

Lot	Cow No.	Initial		Dam [a]
		Age *(wk.)*	Weight *(lb.)*	
I	1	7	179	14
	2 rg [b]	6	122	....
Control	3 (R) [c]	26	504	10
	4	20	383	2
	25 rg	6	138	....
II	5	6	161	....
	6	20	405	5
1.0 mg. F/kg	7	25	470	16
	8 rg	8	200	....
	9 rg (R)	17	300	31
III	27 rg	20	387	....
	10 (R)	6	199	....
1.2 mg. F/kg	11	24	500	27
	28	20	315	....
	12 rg (R)	8	181	....
	13 rg	22	403	33
IV	14 rg (R)	14	309	4
1.4 mg. F/kg	15	21	400	29
	16	22	417	33
	17	8	181	....
	18	21	410	15
V	19	18	342	17
1.6 mg. F/kg	20	10	237	....
	21	6	164	....
VI	22 (R)	21	429	30
2.0 mg. F/kg	23 (R)	6	157	....
	24	27	515	22

[a] Number (in Experiment 809A or 809B) of the dam of this animal.
[b] rg = restricted grain.
[c] (R) = continued on recovery study, 809 R.

bone), and frontal bone, twelfth rib, and vertebrae (as examples of cancellous, more metabolically active bones). The maxillae and mandibles with full dentition were likewise retained for study and analyses. Bones were cleaned by boiling in a distilled water detergent solution, and air-dried prior to examination.

RESULTS

The level of fluorine ingested had no effect upon the general health of the cattle, and mastitis was not the serious problem it had been in the two previous studies. Calf No. 26, which would have been a reduced grain intake control, was removed from the herd as a brucellosis suspect early in the experiment.

Five calves were born with stiffened front legs, a condition not correlated with fluorine ingestion, frequently observed among newborn foals and calves. Cow No. 2[illegible] aborted a 4-mo. fetus in her first gestation, and No. 24 a 6-mo. fetus in her third gestation. Cows which were bred, diagnosed as pregnant, but which had to be rebred were No. 7, 28, 15, 17, 20, 23, and 24. The over-all breeding efficiency of the herd was slightly over two services per conception—below

normal. Many factors can influence breeding performance and in this experiment there was no apparent effect of fluorine ingestion on this factor.

The milk production records are presented in Table 2. Complete data are available for only the first lactation, as many of the cows were slaughtered before completing the second. Production of the herd was low. The individual production varied considerably and no direct effect of level of fluorine ingestion on milk production could be demonstrated, even though the cattle of Lot VI (2.0 mg. F/kg) demonstrated moderate systemic evidence of fluorosis in the first lactation and more severe symptoms in the second. The initial age of exposure, ranging from 6 to 27 wk., had no effect on milk production. Likewise, reducing the grain intake had no effect on milk production in this experiment. The observation that fluorine did not interfere with milk production is in line with results previously obtained with 2-yr.-old heifers (11) or mature cows (13).

TABLE 2
Effect of fluoride ingestion on milk production

Lot	Cow No.	1st lactation			2nd lactation		
		Days	Milk	Fat	Days	Milk	Fat
			(lb.)			(lb.)	
I Control	1	295	5,346	200	242	6,018	224[a]
	2	337	8,610	308	159	6,517	229[a]
	3	342	8,951	330	393	11,229	409
	4	281	6,563	245	246	8,666	259
	25	541	14,293	518[a]	.....	.....	.....
II 1.0 mg. F/kg	5	294	7,872	299	227	7,932	286[a]
	6	335	9,636	370	162	5,603	205[a]
	7	217	7,243	272	466	10,223	349[a]
III 1.2 mg. F/kg	8	341	8,802	343	172	6,595	237
	9	300	7,955	252	367	10,218	307
	27	309	8,227	308[a]	.....	.....	.....
	10	299	7,034	275	296	8,156	277
	11	230	4,672	159	240	4,778	145
	28	366	11,064	432[a]	.....	.....	.....
IV 1.4 mg. F/kg	12	320	8,581	296	276	7,097	255
	13	165	2,353	79	136	2,179	78
	14	348	6,940	276	280	7,539	286[a]
	15	350	5,922	253	227	7,460	231
	16	300	7,183	256	.....	.....	.....[b]
	17	334	10,346	370	231	7,320	260[a]
V 1.6 mg. F/kg	18	298	7,224	250	270	6,147	213
	19	325	8,427	317	269	8,135	283[a]
	20	307	6,542	261	.....	.....	.....[b]
	21	322	7,898	313	217	5,721	209[a]
VI 2.0 mg. F/kg	22	202	6,837	231	306	5,164	161
	23	425	9,518	254	.....	.....	.....
	24	340	8,474	329	345	8,491	312[a]

[a] Slaughtered before completing lactation.
[b] Slaughtered before start of second lactation.

The effects of added increments of dietary fluoride upon body weights are presented in Table 3. Examination of the data show that a reduced grain intake resulted in a body weight (at 80 wk.) approximately 100 lb. less than

those fed the normal dairy cow ration. Supplemental increments of fluoride up to 2 mg/kg of body weight had no effect upon body weight gain or the rate of growth. Weights of these cattle are in the same range as those previously recorded for the cows in Experiment 809A (12), which were not exposed to increased dietary fluorine until 2 yr. of age. It would appear, from this comparison, that a moderate curtailment of food intake can have a more pronounced effect on body weight than an early exposure to relatively high levels of fluorine intake. The data in Table 3, summarizing the post-partum weight loss, indicate that during the first lactation there was little or no effect of fluoride ingestion on maintenance of body weight through early lactation.

TABLE 3
Effect of fluoride ingestion on body weight (average by lots)

Lot	Weeks on experiment			Post-partum weight loss 1st lact.
	24	56	80	
	(lb.)			*(% of max. weight loss)*
I Control	416	865	1,095	17
Restricted grain	365	783	988	14
II 1.0 mg. F/kg	415	839	1,093	21
III 1.2 mg. F/kg	447	835	1,066	17
Restricted grain	414	783	966	19
IV 1.4 mg. F/kg	423	860	1,099	15
Restricted grain	421	790	986	17
V 1.6 mg. F/kg	430	811	1,047	17
VI 2.0 mg. F/kg	425	823	1,068	20

Chemical analyses provide one of the most definitive and reliable tests in diagnosis of fluoride toxicosis. Data on tail vertebrae biopsy samples and urine fluorine concentration are presented in Table 4. While the control cows excreted less than 5 p.p.m. F in the urine, those cows which were on a borderline of toxicity by other criteria excreted 20-30 p.p.m. F and cows showing systemic signs of toxicity excreted over 35 p.p.m. F. It can be seen that the fluorine content of the vertebrae biopsies after 18 mo. of exposure represented from 70-90% of that obtained by 30 mo. This decline in retention rate as the fluorine

TABLE 4
Effect of fluoride ingestion on fluorine content of urine and bone biopsies

Lot	Tail vertebrae		Urine	
	18 mo.	30 mo.	'57-'58	'59-'60
	(p.p.m. F, ash wt.)		*(p.p.m. F, SPG 1.04)*	
I Control	281 ± 55[a]	374 ± 48	3.3	2.4
II 1.0 mg/kg	4,730 ± 590	5,030 ± 480	18.7	17.5
III 1.2 mg/kg	5,050 ± 370	6,070 ± 720	17.6	23.0
IV 1.4 mg/kg	5,290 ± 620	6,910 ± 1,190	22.9	29.8
V 1.6 mg/kg	6,060 ± 140	8,920 ± 860	25.6	37.0
VI 2.0 mg/kg	7,830 ± 200	9,760 ± 1,100	37.0	48.0

[a] Standard deviation.

exposure period was extended is characteristic of fluorine metabolism, and was probably responsible for the increase in urinary fluorine as the study continued.

Results of fluorine analysis of representative tissues removed at slaughter are presented in Table 5. The fluorine content of the skeleton increases with

TABLE 5

Effect of fluoride ingestion on tissue fluorine content

	Rib (p.p.m. F, Ash wt.)	Vertebrae (p.p.m. F, Ash wt.)	Frontal (p.p.m. F, dry fat-free wt.)	Metacarpus (p.p.m. F, dry fat-free wt.)	Liver (p.p.m. F, dry wt.)	Kidney (p.p.m. F, dry wt.)
I Control (4)[a]	384	461	214	206	1.0	1.3
II 1.0 mg. F/kg (3)	6,550	8,300	4,780	4,040	1.3	7.0
III 1.2 mg. F/kg (4)	6,390	7,620	4,860	3,860	1.4	5.6
IV 1.4 mg. F/kg (4)	8,480	8,950	5,560	4,790	1.1	6.1
V 1.6 mg. F/kg (4)	11,030	11,740	7,350	6,020	1.3	7.1
VI 2.0 mg. F/kg (1)	13,100	13,900	8,220	7,200	2.4	8.4

[a] Numbers in parentheses represent number of cows averaged. Table 1 indicates cows continued on experiment.

increased dietary fluoride and varied with the type of bone involved. The cancellous vertebrae, frontal, and rib bones, which have a more active metabolism and along with newly formed trabeculae constitute the readily available Ca and P pool for lactation, retained more fluorine than the compact, more stable, metacarpal bone. Differences in starting weight, age, or fluorine exposure history of the dam had no apparent effect on terminal fluorine retention. Because of the small increase caused by fluorine ingestion, the soft tissue fluorine concentration was found to be unreliable as a measure of degree of fluorosis.

In Experiment 809A (12) a systemic reaction to fluoride ingestion was noted in certain of the cattle, and it was found to be associated with a skeletal fluorine level of 5,500 p.p.m. (dry fat-free weight) in the long bones. This reaction was characterized by a rapid loss of weight and a general deterioration of condition, accompanied by an intermittent lameness and stiffness. The data showing the relationship between skeletal fluoride level and systemic toxicity symptoms, and the relationship of fluorine level to the time interval needed to produce toxicity, are summarized in Table 6. Cows in Lot VI exhibited temporary systemic effects in their first lactation. During the second lactation the symptoms were severe. Analytical data were available on Cow 24 only since

her lot-mates were transferred to the recovery experiment. Meanwhile, cows in Lot V reached toxic levels in their second lactations which were reflected and verified by the bone fluorine concentration. The active cases demonstrated fluorine concentrations in excess of 5,500 and 7,000 p.p.m. (dry fat free weight) in the metacarpal and frontal bone, respectively. As the ash weight of the bone is about 70% of the dry fat free weight, it can be seen that the fluorine content of the vertebrae is very similar to the frontal bone and higher than the metacarpus.

TABLE 6

Bone fluorine retention in relationship to fluorine toxicity

Cow No.	Appearance of systemic reaction	Bone fluorine content		
		Metacarpus p.p.m. F (dry fat-free wt.)	Frontal	Vertebrae p.p.m. F (ash wt.)
20 mg. F/kg				
22	First lactation (PLS)[a]	.....[b]	.....	.....
23	First lactation (PLS)	.....	.....	.....
24	First lactation (PLS)	7,200	8,200	13,900
1.6 mg. F/kg				
18	Second lactation (PLS)	5,630	7,140	10,750
19	Second lactation (PLS)	5,760	7,270	11,250
20	Second lactation (PLS)	6,030	7,280	12,500
21	Second lactation (PLS)	6,670	7,700	12,450
1.4 mg. F/kg				
12	None	.....	.....	.....
13	Mild, if any	3,970	5,250	8,200
14	Second lactation (LS)	.....	.....	.....
15	None	5,200	5,930	9,480
16	None	4,850	5,500	8,650
17	Mild in second lactation (LS)	5,130	.....	9,500

[a] Condition of animal: gaunt or pinched, lame, and stiff.
[b] Animal not slaughtered.

The effects of fluorine upon the incisor teeth of these animals was followed by examination during the course of the study and a final grading at the end. According to Hobbs (3), a score up to 4 indicates little to slight mottling, while a score of 4 indicates extensive mottling with suggestive hypoplasia of the enamel. A score of 5, 5a, 5b, and 5c classifies the degree of hypoplastic enamel and wear of the incisors. In this experiment both Lot V and Lot VI cattle developed incisors classified 4 and 5 in the first pair, 5a and 5b for the second and third pairs, and a 5c score for the fourth pair. In Lot IV the majority of teeth of the third and fourth pair scored 5, whereas the first and second pair incisors were classified 3 or 4. Most of the incisors from the cows in Lots II and III were classified 2 or 3, with an occasional score of 4 or 5 for the third and fourth pair of incisors. The development, in some cows, of fluorine-affected incisors with no other supporting criteria of fluorine toxicity again points out the extreme sensitivity of the developing tooth to fluorine and other factors. Perhaps a more serious effect, since it influences mastication, was a softening of the molars, leading to an increase in molar wear of

the cows in Lots V and VI. This excessive wear amounted to about ¾ in. in some cows, and such animals persistently resorted to lapping water from the cup rather than drinking in a normal manner.

Changes in the bone structure of the animals fed elevated levels of fluorine have been well-documented (2, 9, 17). When the bones were cleaned after slaughter, fluorotic effects were evident in some animals from each of the fluoride-fed lots. There was a definite increase in the severity of the lesions at and above an ingestion level of 1.4 mg. F/kg (Lot IV). Exostoses were variable, and resulted in thick, rough bones, particularly in Lots V and VI. In these lots, calcification at the proximal end of the metatarus tended to ankylose. In these latter groups the general size of the bone was increased, and frequently the bones of the skull were distorted. Most easily observed were thickened mandibles which twisted outward, thus flattening the incisoral arch. Eventually, the bones of the face arched upward, giving a Roman nose effect to the silhouette. At the lower ingestion levels, the only effects noted were a slight calcification of the capsular ligaments of the hock, and thin diffuse areas of periosteal bone on the shaft of the metatarsus. The metacarpus was affected to a lesser degree. Only the advanced cases of exostosis in Lots V and VI were of significance in diagnosis or detectable prior to slaughter.

DISCUSSION

Previous studies indicated that a level of fluorine in the long bones in excess of 5,500 p.p.m. (dry fat-free weight basis) was indicative of and correlated with the appearance of systemic effects of fluorine toxicosis. Cows in this range also exhibited changes in the skeleton, excreted over 30 p.p.m. of fluorine in the urine, were susceptible to lameness and appetite repression and, if exposed to fluorine early enough, possessed badly mottled teeth of inferior wearing quality. It was suggested that the range of 4,500 to 5,500 p.p.m. might be considered the marginal zone of toxicosis and that levels below this were not harmful. In this experiment, the cows receiving 1.6 or 2.0 mg. F/kg had in excess of 5,500 p.p.m. F in the metacarpus, and those in Lot IV (1.4 mg. F/kg) were in the 4,000-5,000 p.p.m. range. The results discussed indicate that the level of 1.4 mg. F/kg was also the dividing above which extensive dental wear and bone involvement were found and systemic effects noted.

In a recent study by Newell and Schmidt (6), 2-yr.-old heifers on pasture were fed up to 2.5 mg. F/kg body weight for an extended period, with only mild physiologic symptoms of fluorosis developing. However, the skeletal fluoride level in those animals receiving the most fluoride was only in what our data should indicate to be marginal range. It has been previously demonstrated (7) that pasture has a mitigating effect on the symptoms of fluorine toxicity. These results are consistent with the viewpoint that the best single measurement of fluorosis is the level of skeletal fluoride, although it should be emphasized that no single symptom is definitive.

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EFFECT OF SOMATOTROPHIN AND OXYTOCIN ON THE MAMMARY GLAND DEVELOPMENT OF GOATS TREATED WITH ESTROGEN AND PROGESTERONE [1]

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SUMMARY

Neither the addition of somatotrophin nor oxytocin to estradiol and progesterone injections caused an increase in the udder alveolar surface area or milk production of virgin yearling goats as compared to estradiol and progesterone alone. The total alveolar surface area and milk production resulting from any combination of hormones used for inducing mammary gland growth was less than half of that of the goats whose udders were developed through pregnancy. All hormone-treated groups showed a deficiency of total alveolar surface area. The correlation of previous 4-wk. milk production and total alveolar surface area was 0.947. Goats injected with growth hormone started out at a higher level of milk production than the two other hormonally induced groups. No histological abnormalities were observed in the hormone-treated groups.

Mammary gland growth and lactation can be induced in sterile cows, virgin heifers, and virgin goats by injecting estrogen and progesterone. The resultant mammary gland growth and milk production are less than those obtained through pregnancy. Estrogen injected alone can cause mammary gland growth and milk production; however, the addition of progesterone will reduce the histological abnormalities observed in goat and heifer udders developed by estrogen treatment alone (2, 5, 17, 23).

Somatotrophin (STH) increases milk production in cows and goats when it is injected during a normal lactation [reviewed by Meites (16)]. Somatotrophin is also required for the duct and lobulo-alveolar development in hypophysectomized rats and mice (1, 6, 10, 14, 15, 19) and appears to have a direct mammogenic effect on duct proliferation (14, 15, 19). Sykes and Wrenn (22, 23) found that pituitary extracts added to estrogen or estrogen and progesterone resulted in a more normal development of the mammary tissue in dairy heifers than injections of the steroids alone.

Oxytocin is involved in the milk-ejection complex. It also retards mammary gland involution in rats after removal of the suckling young, as first shown by Benson and Folley (3). Staples *et al.* (21) observed that heifers treated with oxytocin for short periods of time showed an increase in mammary gland size and noted the presence of secretory alveoli in the glands of these animals.

The object of this experiment was to determine whether somatotrophin or

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oxytocin added to estradiol and progesterone would increase the mammary gland development and milk production resulting from estradiol and progesterone treatments alone in dairy goats.

EXPERIMENTAL PROCEDURE

Twenty-eight virgin yearling does were divided into four groups. The goats in Group I were bred and five of the seven in this group kidded normally. The remainder of the goats received 140 mg. progesterone and 1 mg. estradiol subcutaneously on the alternate days. The seven goats in Group II received estradiol and progesterone injections only. The goats in Group III received estradiol and progesterone plus 14 mg. somatotrophin [2] on alternate days, whereas the goats in Group IV received 50 U.S.P. units of oxytocin [3] daily in addition to estradiol and progesterone. All injections were continued for 150 days. Milking was begun on the day after the last injection. No triggering dose of estradiol was used, since Benson *et al.* (2) have shown it to have no effect on milk production with the hormone dosages used in this experiment.

Daily milk weights were obtained for 8 wk. At this time one-half of each udder was removed and fixed by the method described by Cowie *et al.* (5). Total surface area of secretory epithelium was determined by the method described by Cowie *et al.* (5) and Richardson (20) and modified by Benson *et al.* (2). Fifteen randomly selected sections from each udder were obtained, of which five were used for density determinations. All 15 sections were used for histological studies. Ten fields of alveolar tissue were counted for each section. These sections were projected at a magnification of 620 times on a screen with a single grid line of 70 cm.

RESULTS

Results of the experiment are given in Table 1, from which it can be seen that the goats whose udders were developed through pregnancy produced slightly more than twice the amount of milk produced by any of the three other groups. The milk produced by this control group was significantly greater ($P < .01$) than the three other groups. No significant differences occurred between the three other groups. The total alveolar surface area measurements followed the same trend as the milk production. This agrees with the work of others (2, 5, 20) indicating that the hormonal induction of mammary gland growth results in an udder markedly deficient in alveolar surface area. Oxytocin and STH did not increase the alveolar surface area or milk production over that obtained by estradiol and progesterone injections.

The asymmetry index (defined as the per cent of milk yielded by the highest producing half) was not significantly different between groups. Benson *et al.* (2) have reported that progesterone added to estrogen reduces the variation in milk production between halves compared to estrogen alone. The porosity index (defined as the alveolar surface area per unit volume of tissue) was signifi-

[2] The somatotrophin was an Armour preparation supplied as a gift from the Endocrinology Study Section, National Institutes of Health.

[3] Armour's P. O. P., kindly supplied by Dr. M. Davenport.

TABLE 1

Effect of somatotrophin and oxytocin on milk production and alveolar surface area of virgin yearling goats injected with estradiol and progesterone

Treatment	No. goats	Milk yield from excised half—28 days	Wt. of excised half	Porosity [a] index	Density of tissue	Alveolar surface area	Asymmetry [b] index
		(lb.)	*(g.)*			*(sq. m.)*	
Pregnancy	5	55.4**	502.6[n.s.]	196**	1.02[n.s.]	9.67**	52[n.s.]
E+P [c]	7	24.5	368.1	117	0.97	4.40	59
E+P+Oxy. [d]	7	16.9	293.4	138	0.99	4.04	61
E+P+STH [e]	7	23.2	345.9	125	0.98	4.40	54

[n.s.] Nonsignificant differences between treatment means ($P > 0.05$).

** Treatment means due to pregnancy significantly different from others ($P < 0.01$).

[a] Porosity index = alveolar surface area per unit volume of tissue, $2n/l$, n = No. alveoli intersecting a line of length l.

[b] Asymmetry index = per cent of milk yield by highest producing half.

[c] 1.0 mg. estradiol and 140 mg. progesterone every other day.

[d] Estradiol, progesterone, and 50 U.S.P. units oxytocin daily.

[e] Estradiol, progesterone, and 14 mg. STH on alternate days.

cantly greater ($P < .05$) in the control group than in the three other groups. No statistically significant differences ($P > .05$) occurred between the other groups. This indicates that the alveoli in hormonally induced udders are much larger than those in the control group. No histological abnormalities, such as cystic alveoli, papillomatous outgrowths from the epithelium, or immature lobules reported by Benson *et al.* (2) for goats treated with estrogen alone, were noted in any of the udders.

The weights of the excised udders for the various groups were not significantly different, although the weights of the control group were higher. This result, combined with a higher porosity index, caused a significant increase in total alveolar surface area in the controls. The density determinations between the four groups were not significantly different.

DISCUSSION

Somatotrophin appears to have some direct effect on ductal and lobulo-alveolar mammogenesis in the rat and mouse (14, 15, 19). It is definitely required for normal mammary gland growth in the hypophysectomized rat and mouse (1, 6, 10, 14, 15, 19). This may be due in part to its ability to restore a normal metabolic state in the hypophysectomized animal, as Jacobsohn (12) and Donovan and Jacobsohn (7) have shown that the growth of the mammary gland is dependent upon the metabolic state of the individual. Somatotrophin also increases the DNA content of the mammary glands of ovariectomized rats when added to estradiol-benzoate, progesterone, and thyroxine (18). Flux (9) also found that STH increased the mammary gland duct growth of ovariectomized mice with or without the injections of estrone. Lucherini and Schiavetti (13) found that the administration of STH to male rabbits caused mammary gland hypertrophy in a high per cent of the treated animals. Somatotrophin in the

amounts used in this experiment did not have a synergistic effect with estradiol and progesterone to increase mammary gland growth.

Oxytocin also had no synergistic effect with estradiol and progesterone in increasing mammary gland growth. In fact, the alveolar surface area was somewhat lower in the oxytocin-treated animals than in the two other groups in which lactation was induced.

The correlation coefficient between previous 4-wk. milk production and total alveolar surface area was 0.947. The regression of 4-wk. milk production was 6.25 lb. milk on each square meter of alveolar surface area. This high correlation coefficient agrees with that obtained by Benson *et al.* (2), Richardson (20), and Goto, Oshima, and Joike (11). The percentage of the total variation in previous 4-wk. milk production accounted for by the alveolar surface area is 89.7 ($R^2 = 0.897$). This result indicates that the major limitation of milk production of goats at the peak of lactation is the alveolar surface area.

To determine whether 15 sections of udder tissue were required to obtain an accurate measure of the alveolar surface area, five sections were selected at random from the 15 and the sections counted. A difference of 1.3% was found

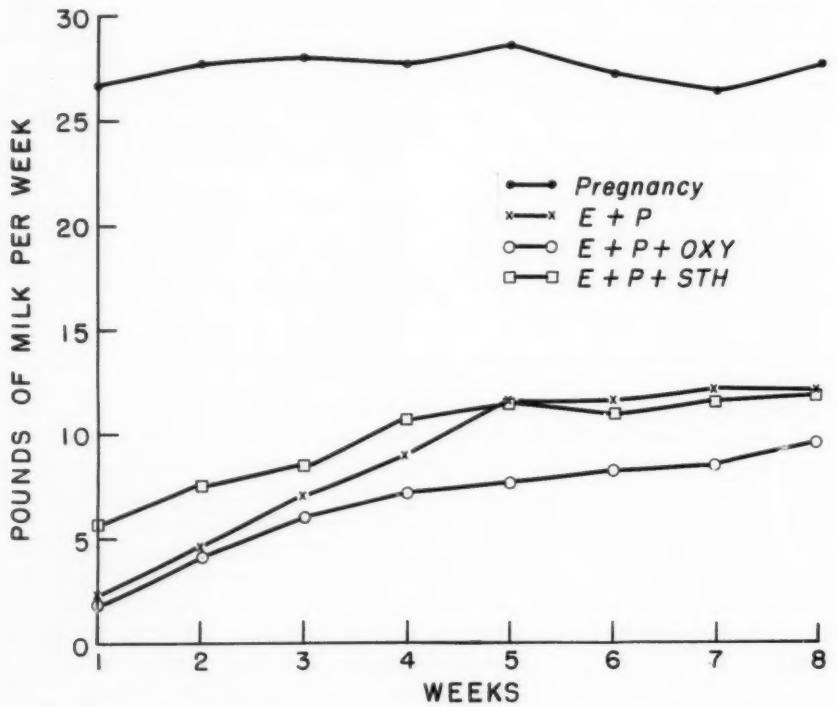

FIG. 1. Weekly milk production of goats whose udders were developed through pregnancy and hormone injections.

between the two methods. The correlation between the previous 4-wk. milk production and alveolar surface area using five sections per gland was 0.934. It would appear that the number of sections per gland can be greatly reduced without markedly decreasing the accuracy of the method.

Somatotrophin appeared to have a stimulatory effect on the initiation of lactation (see Figure 1). It will be noted that goats receiving STH started lactation at a much higher level of production than the two other hormonally induced groups. At 5 wk. after the onset of milking the estradiol-progesterone treated group produced the same amount of milk as the group which received STH in addition. The group receiving oxytocin started lactation at about the same level as the estradiol-progesterone-treated group, but did not reach production level of the other groups. That STH may be involved in the initiation of milk secretion is supported by the work of Folley, Malpress, and Young (8), who found that ox pituitary hormone added to estrogen caused the goats to come into lactation sooner than estrogen alone and Chung (4), who found that one member of a pair of identical twin cows injected with STH before parturition produced more milk than its mate.

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THYROXINE TURNOVER METHODS FOR DETERMINING THYROID SECRETION RATES IN DAIRY CATTLE [1, 2]

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SUMMARY

Various radiothyroxine and normal thyroxine turnover methods for rapid determinations of thyroid secretion rate were compared within animals. The preferred radiothyroxine method was an isotope dilution procedure based on the decline in specific activity of PBI following the injection of I^{131}-labeled L-thyroxine. The preferred normal thyroxine turnover method was a chemical procedure based on the decline in plasma PBI after the injection of nonradioactive thyroxine. Daily thyroid secretion rates (per 100 lb. body weight), determined by the isotope dilution and chemical PBI methods, respectively, averaged 0.40 and 0.39 mg. in 14 young bull calves and 0.14 and 0.13 mg. in four nonlactating cows. In the cows, daily thyroid secretion rates (per 100 lb. body weight), determined by the commonly used thyroxine replacement method based on the inhibition of thyroidal-I^{131} release, averaged 0.13 mg. A simple correlation coefficient of 0.93 was found between thyroid secretion rates determined by the isotope dilution and the chemical thyroxine turnover methods in bull calves. Error coefficients of variation of thyroid secretion rates obtained from duplicate determinations in four cows were 9.7 and 11.5% for the isotope dilution and chemical thyroxine turnover methods, respectively. Evidence is presented that the turnover rate of I^{131}-labeled L-thyroxine is not influenced significantly by the recycling of I^{131} through the thyroid during the first 96 hr. after radiothyroxine injection or by the single large dose of normal thyroxine used in the chemical thyroxine turnover method.

The estimation of thyroid secretion rate offers one of the best measures of thyroid gland activity in dairy cattle. In the method used most extensively in domestic animals, thyroid secretion rate is determined as the minimum amount of thyroxine administered daily that will completely block the release of previously injected I^{131} taken up by the thyroid gland (2, 4, 5, 10, 11, 15). Sorensen and Moustgaard (20, 21) proposed a different method, in which the release rate of thyroidal-I^{131} and the specific activity of the circulating thyroid hormone were used for secretion rate calculations. Both of these methods require considerable time (2 wk. or more) and are subject to the restrictions imposed by the use of radioisotopes.

Demonstration of the equivalence between the amount of thyroxine degraded by the body per day and that synthesized by the body per day (7) indicates that thyroxine degradation rates may be used as estimates of thyroid secretion

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[2] This study was part of a Northeast Regional Research Project (NE-41, Endocrine Factors Affecting Reproduction and Lactation in Dairy Cattle), a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds of the United States Department of Agriculture.

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rates. The use of I^{131}-labeled thyroxine turnover techniques (3, 7, 22, 23) has enabled the determination of thyroxine degradation (or secretion) rates in only a few days' time. Mixner and Lennon (12, 13) have proposed an adaptation of the radioactive thyroxine turnover technique in which thyroid secretion rate is determined from the turnover of normal thyroxine, thus eliminating the need for radioisotopes.

The purpose of this paper is to present a new radiothyroxine turnover method for obtaining more direct estimates of thyroid secretion rates based on the isotope dilution procedures of Peterson and Wyngaarden (14), as used with hydrocortisone and the adrenal cortex, and to evaluate and further develop the nonradioactive (chemical) thyroxine turnover method of Mixner and Lennon (12, 13). Also presented are comparisons among these methods and the radiothyroxine degradation rate method of Freinkel and Lewis (3) and the thyroxine replacement method based on the thyroxine inhibition of the thyroidal-I^{131} release. Preliminary reports have been made on this study (16, 17).

MATERIALS AND METHODS

Thyroid secretion rates were determined in 14 bull calves (six to 30 days of age) and four mature, nonlactating cows of the Holstein and Guernsey breeds. For radiothyroxine turnover methods a single tracer dose (50 μc. in calves, 250 μc. in cows) of I^{131}-labeled L-thyroxine (Abbott Laboratories) in aqueous solution was injected intravenously into the jugular vein. During the degradation phase, after complete distribution of the labeled hormone, blood samples were collected approximately 24, 48, 72, and 96 hr. after injection. For the isotope dilution method of secretion rate determination the specific activity of plasma protein-bound iodine (PBI) was determined in each blood sample. Plasma PBI was measured by the method of Brown *et al.* (1), as modified by Lennon and Mixner (9). The radioactivity associated with PBI was measured in the dissolved ashed plasma proteins just prior to the final colorimetry step in the PBI procedure with a well-type scintillation counter. Thus, the accuracy of the specific activity measurements (counts per minute per microgram of PBI) would be independent of any iodine losses during protein precipitation, washing, drying, and ashing in the PBI determination. Indeed, using the specific activity method, it would not be necessary to measure accurately the amounts of plasma to be used for the combined PBI and radioactivity measurements. For other radiothyroxine secretion rate methods, radioactivity was measured in whole plasma and in the precipitated and washed plasma proteins.

The chemical thyroxine turnover method of determining thyroid secretion rate was initiated immediately following the radiothyroxine methods, with the single intravenous injection of normal thyroxine (5 mg. in calves, 25.2 mg. in cows) in the form of sodium L-thyroxine pentahydrate[4] (mol. wt.—888.995, purity—91.5%). Blood samples were collected approximately 24, 30, 48, 54, and 72 hr. after injection and assayed for plasma PBI. Measurements of

[4] Supplied through the courtesy of Dr. H. L. Saunders, Smith, Kline, and French Laboratories, Philadelphia.

plasma-I^{131}, plasma protein-I^{131}, and the specific activity of PBI were continued during the chemical method, to evaluate the influence of excess thyroxine on thyroxine turnover rates.

Each bull calf was subjected to one trial in which thyroid secretion rate was determined by both radiothyroxine and normal thyroxine turnover methods. Each cow was subjected to two such trials, one month apart. One month after the second thyroxine turnover trial in each cow, thyroid secretion rate was also determined by the thyroxine replacement method, based on the thyroxine inhibition of thyroidal-I^{131} release.

For studies of the thyroid recycling of I^{131}, six additional bull calves were each injected with 50 μc. of I^{131}-labeled L-thyroxine. Two received no other treatment, two received 0.2 g. of thiouracil per kilogram of body weight orally per day, and two were fed 250 mg. of sodium iodide per day. Blood samples collected at 24-hr. intervals were assayed for whole plasma radioactivity.

RESULTS AND DISCUSSION

Thyroxine turnover data obtained from a typical bull calf (H771[9]) after single injections of radioactive and normal L-thyroxine were plotted semilogarithmically (Figure 1). The turnover of I^{131}-labeled L-thyroxine (I^{131}-T_4) is represented by the disappearance of radioactivity from whole plasma and from the precipitated plasma proteins and by the decline in specific activity of plasma PBI. The turnover of normal L-thyroxine (I^{127}-T_4) is represented by the decline in total plasma PBI values and by the decline in PBI response (total PBI minus preinjection PBI). All the curves were considered to be exponential in nature and to be represented by the regression equation; $Y = ae^{-bX}$, which may be transformed to the linear logarithmic form $ln\ Y = ln\ a - bX$. Where $Y =$ plasma I^{131}, plasma protein I^{131}, specific activity of PBI; and total PBI or PBI response to injected normal thyroxine

$X =$ time in hours

$a = Y$-axis intercept (value of Y when $X = 0$)

$b =$ regression coefficient or thyroxine turnover rate per hour.

Each thyroxine turnover curve was used for calculating a separate estimate of thyroid secretion rate. According to the method of Freinkel and Lewis (3), the plasma-I^{131} curve and the normal level of plasma PBI were used in calculating the thyroxine fractional turnover rate (TFTR), the thyroxine volume of distribution (TVD), and the circulating level of thyroxine. Thyroid secretion rate (TSR) was obtained as the product of these three components.

The same type of calculations were made from the plasma-PBI^{131} curve to obtain a second estimate of thyroid secretion rate. This might be considered a more accurate estimate, since PBI^{131} is more representative of plasma thyroxine-I^{131}. Both the plasma I^{131} and the plasma PBI^{131} methods actually measure the amount of thyroxine being degraded or eliminated from the extrathyroidal thyroxine pool per unit time. However, under steady state conditions of constant pool size, the amount of thyroxine being eliminated from the pool must equal that being secreted into the pool.

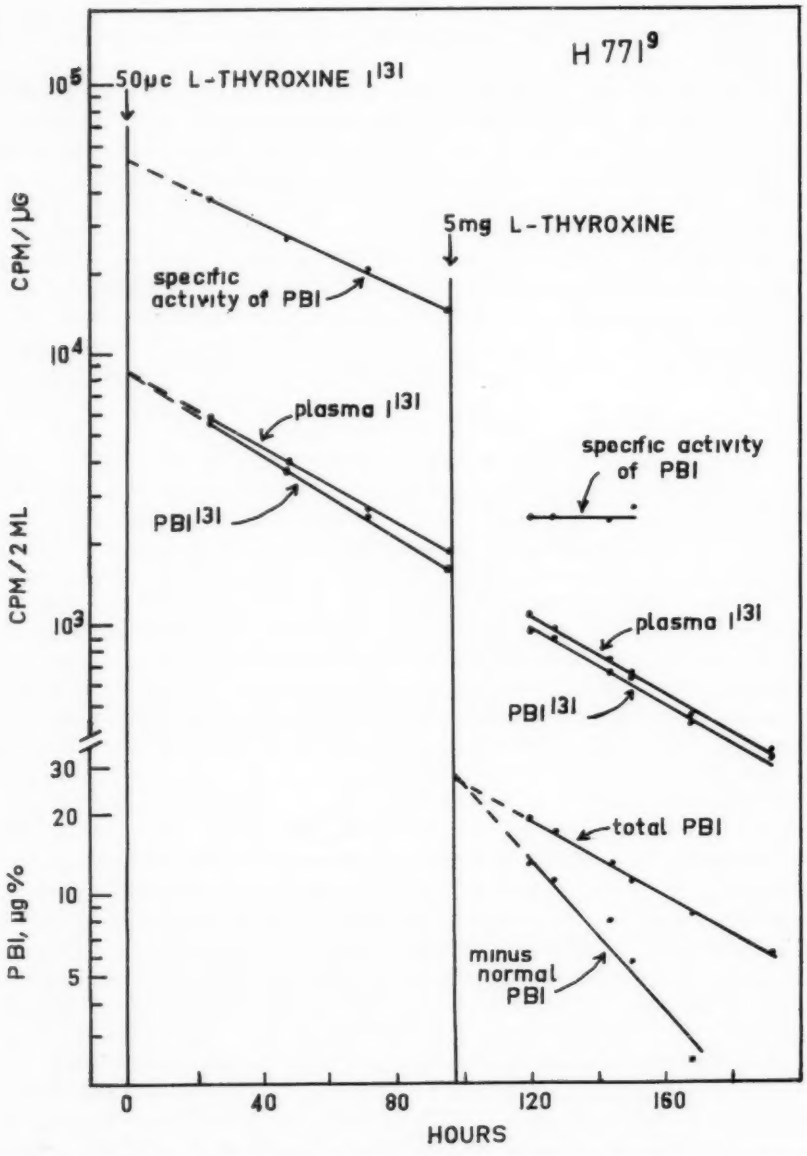

FIG. 1. The turnover of single intravenous injections of radioactive and normal thyroxine in a bull calf. Radiothyroxine turnover is represented by the plasma-I^{131}, plasma protein-I^{131}, and specific activity of PBI disappearance curves. Stable thyroxine turnover is represented by the total PBI and total PBI minus normal PBI disappearance curves.

A more direct and preferred radiothyroxine turnover method was an isotope dilution procedure based on the decline in the specific activity of plasma PBI. The rate of dilution of the labeled thyroxine by newly secreted hormone (thyroxine fractional turnover rate) was calculated as the b value in the regression equation (see above) describing the specific activity curve. The extrapolated value of the specific activity at zero-time (a value in regression equation representing the theoretical specific activity at time of thyroxine injection after complete distribution, but before dilution by thyroid secretion) was used in calculating the size of the extrathyroidal thyroxine pool (ETT) according to the formula:

$$\text{ETT, mg.} = \frac{\text{radioactivity injected, c.p.m.}}{\text{Sp. Act. of PBI at 0-time, (c.p.m/}\mu\text{g PBI} \times 0.653 \times 1{,}000)} - \text{mg. of thyroxine-I}^{131}\text{ injected}$$

The constant, 0.653, is used to convert iodine to its equivalent as thyroxine. The product of the thyroxine pool size (ETT) and the thyroxine fractional turnover rate (TFTR) represents the amount of thyroxine entering the pool per unit time or thyroid secretion rate. By dividing the extrathyroidal thyroxine pool by the normal level of thyroxine (from plasma PBI), the volume of distribution of thyroxine was obtained.

As in the method of Freinkel and Lewis (3), daily thyroid secretion rates based on the turnover of normal thyroxine were calculated as products of the thyroxine fractional turnover rate per day, the thyroxine volume of distribution (liters), on the normal preinjection plasma PBI (microgram per cent) divided by 65.3. The thyroxine fractional turnover rate was calculated as the regression coefficient in the previously mentioned equation describing the disappearance of plasma PBI. The thyroxine volume of distribution (TVD) was obtained by the formula:

$$\text{TVD, liters} = \frac{\text{Dose of thyroxine, mg.} \times 100}{a/0.653}$$

where a (Y-axis intercept) equals the theoretical concentration of PBI (microgram per cent) at zero time. Since it was not known whether the decline in total PBI or in PBI response more accurately reflected thyroxine fractional turnover rate, three different secretion rate calculations were made from the PBI data: one based on the decline in total PBI, one based on the decline in net PBI response above normal preinjection PBI (total PBI minus normal PBI), and one based on PBI response values obtained by subtracting the asymptotic PBI level (C_{eq}) approached by the regressing total PBI curve from the total PBI values. The C_{eq} was calculated by the graphic techniques proposed by Hlad *et al.* (6). The mean values of thyroid secretion rates and component measures calculated by the various thyroxine turnover methods in single trials in 14 dairy bull calves are presented in Table 1. The mean results of similar duplicate determinations in four mature, nonlactating cows and of single determinations of secretion rate by the thyroxine inhibition of thyroidal I^{131} release method appear in Table 2.

TABLE 1

Mean thyroid secretion rates and component values as determined by thyroxine turnover methods in dairy bull calves

Characteristic	Thyroxine turnover method					
	Radiothyroxine turnover			Chemical thyroxine turnover		
					PBI response	
	Plasma-I^{131}	Plasma protein-I^{131}	Isotope dil., sp. act. of PBI	Total PBI	Above normal PBI	Above C_{eq}
No. of animals	14	14	14	14	14	14
Age *(days)*	18 ± 2[a]	18 ± 2[a]	18 ± 2[a]	22 ± 2[a]	22 ± 2[a]	22 ± 2[a]
Body wt. *(lb.)*	107 ± 5.7	107 ± 5.7	107 ± 5.7	111 ± 6.2	111 ± 6.2	111 ± 6.2
Dose of I^{131}-T_4 *(μc.)*	50	50	50			
Dose of I^{127}-T_4 *(mg.)*				5	5	5
Normal PBI *(μg. %)*	6.61 ± .52	6.61 ± .52	6.61 ± .52	6.61 ± .52	6.61 ± .52	6.61 ± .52
C_{eq} *(μg. %)*						6.60 ± .83
TFTR per day	.351 ± .014	.387 ± .020	.379 ± .021	.401 ± .015	.757 ± .032	.801 ± .064
Thyroxine $t_{1/2}$ *(hr.)*	48.5 ± 2.2	44.4 ± 2.3	45.7 ± 2.6	42.2 ± 1.6	22.4 ± .8	22.3 ± 1.5
TVD						
Liters	9.42 ± .47	9.07 ± .51	10.58 ± .93	9.84 ± .74	9.70 ± .97	9.38 ± .85
Per cent of BW	20.2 ± 1.3	19.1 ± 1.2	22.0 ± 1.6	20.1 ± 1.7	19.7 ± 2.0	19.1 ± 1.8
ETT *(mg.)*	.986 ± .129	.935 ± .104	1.086 ± .131	1.001 ± .104	1.008 ± .140	.956 ± .108
Daily TSR						
Total *(mg.)*	.344 ± .041	.366 ± .046	.401 ± .048	.386 ± .051	.764 ± .108	.793 ± .130
mg/100 lb. BW	.328 ± .012	.350 ± .014	.383 ± .044	.358 ± .032	.697 ± .096	.719 ± .110
mg/500 kg. BW^{-7}	1.808 ± .202	1.931 ± .232	2.114 ± .240	2.055 ± .168	3.905 ± .540	4.041 ± .624

[a] Standard error of the mean.

TABLE 2

Mean thyroid secretion rates and components as determined by various methods in mature nonlactating dairy cows

Characteristic	Thyroid secretion rate method						
	Radiothyroxine turnover			Chemical thyroxine turnover			
					PBI response		
	Plasma-I^{131}	Plasma protein-I^{131}	Isotope dilution, sp. act. of PBI	Total PBI	Above normal PBI	Above C_{eq}	Thyroxine inhibition of thyroidal-I^{131} release
No. of animals	4	4	4	4	4	4	4
Trials/animal	2	2	2	2	2	2	1
Body wt. *(lb.)*	1,213 ± 53[a]	1,213 ± 53[a]	1,213 ± 53[a]	1,213 ± 53[a]	1,213 ± 53[a]	1,213 ± 53[a]	1,325 ± 43[a]
Dose							
I^{131}-T_4 *(μc.)*	235	235	235				
I^{127}-T_4 *(mg.)*				25.2	25.2	25.2	
NaI^{131} *(μc.)*							250
Normal PBI *(μg. %)*	5.26 ± .40	5.26 ± .40	5.26 ± .40	5.26 ± .40	5.26 ± .40	5.26 ± .40	
C_{eq} *(μg. %)*						4.70 ± .29	
TFTR per day	.369 ± .022	.368 ± .020	.358 ± .019	.396 ± .026	.702 ± .049	.720 ± .097	
T_4 $t_{1/2}$ *(hr.)*	46.3 ± 2.9	46.3 ± 2.8	47.4 ± 2.6	43.3 ± 2.8	24.6 ± 1.9	26.8 ± 4.27	
TVD							
Liters	54.7 ± 3.5	57.3 ± 3.4	62.4 ± 3.5	52.4 ± 3.5	47.7 ± 3.0	46.2 ± 3.49	
Per cent of BW	10.0 ± .62	10.5 ± .65	11.4 ± .68	9.6 ± .78	8.7 ± .59	8.4 ± .62	
ETT *(mg.)*	4.34 ± .32	4.53 ± .30	4.94 ± .48	4.10 ± .21	3.77 ± .24	3.68 ± .36	
Daily TSR							
Total *(mg.)*	1.56 ± .080	1.63 ± .068	1.73 ± .067	1.59 ± .066	2.58 ± .129	2.43 ± .184	1.70 ± .121
mg/100 lb. BW	.130 ± .0068	.136 ± .0067	.144 ± .0066	.134 ± .0089	.219 ± .0130	.203 ± .019	.129 ± .0061
mg/500 kg. BW^{-7}	1.49 ± .073	1.55 ± .067	1.65 ± .065	1.53 ± .089	2.46 ± .130	2.34 ± .205	1.50 ± .065

[a] Standard error of the mean.

In comparing the various thyroxine turnover methods, the isotope dilution procedure was used as a standard, because it was assumed to be the most accurate on theoretical grounds. It measures thyroxine secretion rather than thyroxine degradation, and it is not influenced by iodine losses in PBI assays. The chemical thyroxine turnover methods based on PBI response above normal PBI and above C_{eq} were eliminated from statistical comparison, because they obviously gave erroneously high estimates of thyroxine fractional turnover rates (TFTR) and, therefore, of thyroid secretion rates (TSR). Although all the other methods were not completely independent of each other, since some of the same PBI values were used in each method, the analysis of variance was considered the best available method of evaluating differences. In both the calves and the cows, no significant differences were found between the thyroxine fractional turnover rates obtained by the standard isotope dilution method and those obtained by any of the other thyroxine turnover methods (plasma-I^{131}, plasma protein-I^{131}, and chemical total PBI). Significant differences were found among methods in estimates of thyroxine volume of distribution and extrathyroidal thyroxine pool in both calves and cows and in estimates of thyroid secretion rate in calves. However, Table 3 indicates that these differences, although consistent enough to be significant, contributed very little to the total variance among estimates. The small differences may be accounted for in part by iodine losses in PBI assays. In the PBI procedure used in this laboratory, iodine losses averaged about 4% (18). Such losses cause low estimates of normal plasma PBI and would result in underestimates of the extrathyroidal thyroxine pool and thyroid secretion rate in the plasma-I^{131} and plasma protein-I^{131} methods. In the isotope dilution method, the volume of distribution would be overestimated, but this is of little importance in this method, since the volume of distribution is not used as a component in calculating thyroid secretion rate.

TABLE 3

Per cent of total variance associated with method, animal, and error effects in thyroid secretion rate determinations in dairy bull calves

	Per cent of variance					
				TSR per day *(mg.)*		
Variance source	TFTR per day	TVD, liters	ETT *(mg.)*	Total	Per 100 lb. BW	Per 500 kg. BW^{-7}
Methods	6.27	4.18	1.44	2.02	1.86	2.16
Animals	47.10	68.92	87.90	91.71	91.61	87.74
Residual	46.63	26.90	10.66	6.27	6.53	10.10
Total	100.00	100.00	100.00	100.00	100.00	100.00

The radiothyroxine (isotope dilution) and chemical thyroxine (total PBI) turnover methods of choice[5] were further compared by correlation analysis

[5] Details of the procedures for calculating thyroid secretion rates by these methods are available from the authors in a mimeographed outline. The outline of the procedure for assaying PBI is also available.

of the bull calf data. Figure 2 shows diagrammatically some zero-order coefficients of correlation between the two thyroid secretion rate methods and among measures within each method. Thyroid secretion rates obtained by the two methods were highly significantly correlated ($r = 0.93^{**}$). A significant relationship ($r = 0.63^{*}$) also existed between thyroxine volumes of distribution as determined by the two different methods. The correlation coefficient (0.42) between thyroxine fractional turnover rates, however, did not reach statistical significance. No comparison of PBI levels was made, because the same PBI assays were used for both methods.

Within each method, thyroid secretion rate was significantly related to normal PBI ($P < 0.01$) and thyroxine volume of distribution. Thyroid secretion rate was negatively related ($r = -0.57^{*}$) to thyroxine turnover rate in the chemical method, but no significant relationship was found in the isotope dilution method.

The isotope dilution and chemical total PBI methods were further evaluated in regard to their precision. From the error variance obtained by the analysis of variance of duplicate thyroid secretion rate trials (1 mo. apart) within cows within each method, and from the means shown in Table 2, standard errors of a single determination and error coefficients of variation were calculated (Table 4). Error coefficients of variation of a single determination of thyroid secretion rate and of its components ranged from 8.5 to 9.7% in the isotope dilution method and from 9.5 to 12.8% in the chemical total PBI method. These values compare favorably with the error coefficient of variation of 15.4% calculated from Sorensen's (20) data on duplicate determinations of thyroid secretion rates by a different method.

A possible source of error in radiothyroxine turnover measurements arises from the thyroidal recycling of I^{131} released during the metabolic breakdown

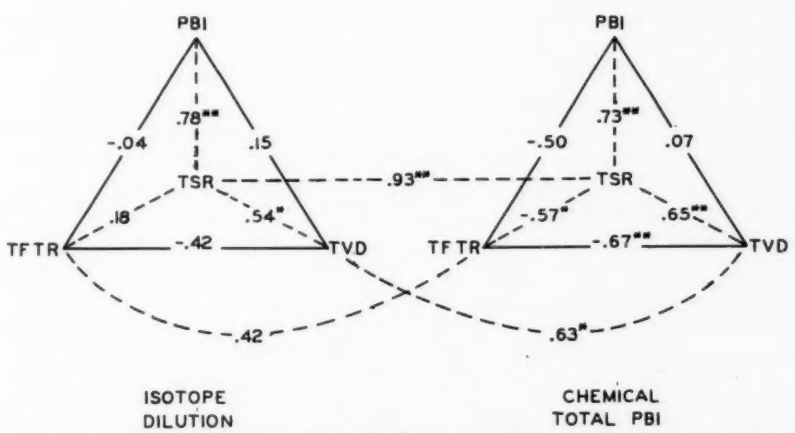

FIG. 2. Diagrammatic presentation of zero-order coefficients of correlation between the isotope dilution and chemical total PBI secretion rate methods and among various measures within each method.

TABLE 4

Estimates of the precision of determining thyroid secretion rates and component values by the isotope dilution and chemical thyroxine turnover methods

Characteristic	Cows			
	Secretion rate method			
	Isotope dilution		Chemical, total PBI	
	S.E.[a]	C.V. (%)[b]	S.E.[a]	C.V. (%)[b]
Normal PBI *(μg. %)*	.448	8.51	.448	8.51
Thyroxine fractional turnover rate per day	.0346	9.66	.0401	10.13
Thyroxine volume of distribution *(liters)*	5.34	8.55	5.32	10.14
Extrothyroidal thyroxine pool *(mg.)*	.471	9.53	.471	11.47
Daily thyroid secretion rate				
Total *(mg.)*	.152	8.78	.204	12.79
per 100 lb. BW *(mg.)*	.0139	9.65	.0153	11.45
per 500 kg. BW^{-7}	.154	9.34	.183	11.94

[a] Standard error of a single determination.
[b] Error coefficient of variation.

of labeled thyroxine. An appreciable amount of recycling would reduce the apparent rate of disappearance of radioactivity from plasma and cause an underestimation of thyroxine fractional turnover rate (3, 7). In the present thyroid secretion rate studies, there appeared to be no influence of I^{131} recycling during the first 96 hr. after the injection of I^{131}-labeled thyroxine, since the thyroxine fractional turnover rates measured from plasma protein I^{131} curves (Figure 1) failed to show any significant increase following a large dose of nonradioactive thyroxine (Table 5). The excess thyroxine effectively blocked thyroid activity and, therefore, the recycling of I^{131}, as evidenced by the leveling off of the PBI specific activity curve (Figure 1). In other bull calves, either thiouracil or excess iodide were also effective in blocking the recycling of I^{131} during the turnover of I^{131}-labeled L-thyroxine, as indicated by a lack of accumulation of radioactivity in the thyroid gland. Like thyroxine, these blocking agents also failed to increase the rate of disappearance of radioactivity from the plasma above that of untreated animals (Table 6). Although the number of animals was too small for adequate comparisons, thiouracil and excess iodide treatments actually appeared to decrease thyroxine turnover rates. Some of the goitrogens have been reported to depress the peripheral deiodination of thyroxine in rats (24). Excess iodide has had similar effects in human Graves disease patients (8).

TABLE 5

Thyroxine fractional turnover rates calculated from plasma protein radioactivity curves before and after a large dose of I^{127}-L-thyroxine

Class of animal	No. of animals	Dose of I^{127}-thyroxine *(mg.)*	Fractional turnover rate/day	
			Before I^{127}-T_4	After I^{127}-T_4
Bull calves	14	5.0	.387	.432
Cows	4	25.2	.368	.362

TABLE 6

Rate of disappearance of I^{131}-labeled L-thyroxine from the plasma of normal bull calves and from those treated with thiouracil or sodium iodide

	No. of animals	Disappearance rate [a]	Half-time [a]
		(per day)	*(hr.)*
No treatment	2	0.394	42.2
Thiouracil 0.2 g/kg body wt/day	2	0.287	58.3
Sodium iodide 250 mg. per day	2	0.277	60.8

[a] Calculated from plasma radioactivity measurements between 24 and 120 hr. after injection of I^{131}-labeled L-thyroxine.

Although the recycling of I^{131} appeared to be of little importance during the first 96 hr. after the injection of I^{131}-labeled thyroxine, it may alter plasma radioactivity at later periods. In bull calves H9-1 (Figure 3) and G23[9], which received no thyroid blocking agents, slower components appeared in the plasma radioactivity disappearance curves after 120 and 168 hr., respectively. At this time, the accumulation of radioactivity in the thyroid region of the neck had reached peak levels.

The chemical thyroxine turnover method might be criticized, because it involves the administration of nonphysiological amounts of thyroxine. However, this study indicates that the single large dose of normal thyroxine did not significantly alter the turnover rate of radioactive thyroxine (Table 5) and did not prevent the accurate measurement of thyroxine turnover and secretion rates by the chemical total PBI method (Tables 1 and 2).

Further evidence for the validity of thyroxine turnover methods was obtained from the similarity of thyroxine secretion rate estimates obtained by them and by the commonly used replacement method based on the thyroxine inhibition of thyroidal-I^{131} release (Table 2). Thyroid secretion rates found in this study, however, were somewhat lower than those found by the replacement method in dairy cattle in other laboratories (11, 19).

Thus, thyroxine turnover methods enable reliable estimates of thyroid secretion rates to be made much more rapidly than by other methods. Of the thyroxine turnover methods, the isotope dilution method is probably the most accurate, but the chemical total PBI method gives comparably good results and may be applied more widely where the use of radioisotopes is restricted.

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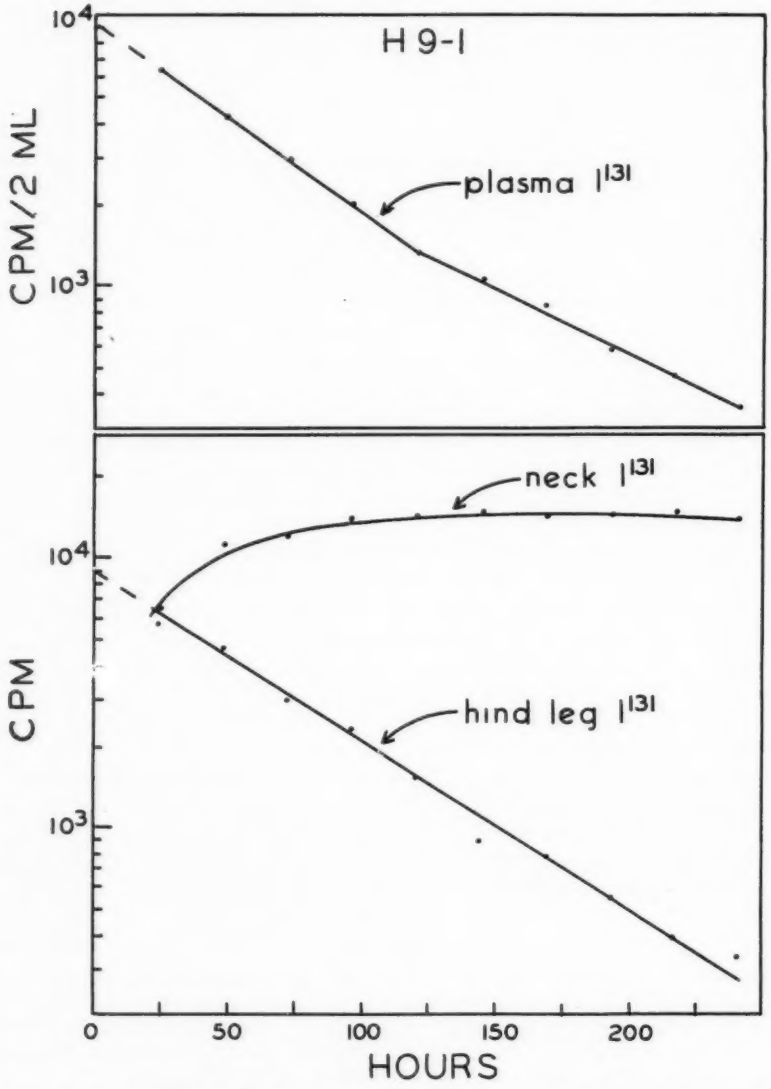

FIG. 3. Radioactivity in the plasma, the thyroid region of the neck, and the upper hind leg of a bull calf following the injection of I^{131}-L-thyroxine.

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EVIDENCE FOR IN VIVO CONVERSION OF C_{21} STEROIDS TO C_{19} ANDROGENS IN THE BOVINE [1]

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SUMMARY

The biosynthetic transformation of progesterone to fecal androgen (androstenedione) in the bovine has been shown. The present study reports the biosynthetic transformation of pregnenolone and 17α-hydroxyprogesterone to fecal androgen. Following the injection of pregnenolone into the male and female bovine, the chick comb response test demonstrated a significant formation of fecal androgens in the male and a significant increase in fecal androgens in the female. Following the injection of 17α-hydroxyprogesterone into the female bovine, androgen biosynthesis was evidenced by the excretion of a significant amount of fecal androgens as measured by the chick comb response test.

In vitro studies with various mammalian tissues have documented a pathway for androgen biosynthesis involving the following sequence: pregnenolone → progesterone → 17α-hydroxyprogesterone → androstenedione. The cumulative results of the experiments on bovine androgenesis provide in vivo evidence indicating that androgen biosynthesis in the bovine, as in many other species, occurs via the same pathway.

The experimental evidence demonstrates further the major importance of an hepato-enteric pathway for the elimination of the C_{19} androgen metabolites of these C_{21} hormones.

Recent research has indicated the important role of progesterone and 17α-hydroxyprogesterone in androgen biosynthesis via the following carefully documented pathway: progesterone → 17α-hydroxyprogesterone → androstenedione (Δ^4-androstene-3,17-dione) → testosterone. Pregnenolone is regarded as the immediate metabolic precursor of progesterone in these transformations.

The in vitro formation of the androgens, androstenedione and testosterone, from progesterone via 17α-hydroxyprogesterone has been demonstrated with rat, bovine, and human testicular tissue (9, 10, 18, 19, 22, 25); bovine ovarian (23); human arrhenoblastoma tissue (20, 27); and hog adrenal preparations (15). In addition, a peripheral biosynthetic mechanism is considered to reside mainly in the liver, which converts certain C_{21} steroids to C_{19} androgens through the action of suitable desmolases. Liver desmolase activity has been shown to convert 17α-hydroxyprogesterone to androstenedione (5).

Various experiments with labeled progesterone have demonstrated that the hepato-enteric pathway is of major importance for the elimination of metabolites of progesterone in the rodent (1, 7, 8, 16, 21) and human (2-4, 6, 17, 26). In addition, large amounts of hitherto unrecognized metabolites of progesterone were indicated in the bile and feces. An earlier investigation indicated the

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importance of the biliary pathway for the elimination of C_{21} metabolites of progesterone in the bovine (14). A recent report demonstrated the presence of hydroxyketonic androgens in the bile of the female bovine (12). These androgens were presumed to be metabolites of progesterone.

In previous reports by the authors concerning steroidogenesis in the bovine, experiments were described which indicated that the androgens found in the feces of the sexually active female bovine are metabolites of progesterone and can be significantly increased upon injection of progesterone (11). The in vivo transformation of progesterone to androgens was also demonstrated in the bull. Following the administration of progesterone to a pregnant cow, the androgens, androstane-3,17-dione, $\Delta^{1,4}$-androstadiene-3,17-dione, and Δ^4-androstene-3,17-dione were isolated from the feces together with etiocholane-3,17-dione (13).

The present study reports in vivo results demonstrating (a) the steroidogenetic role of pregnenolene and 17α-hydroxyprogesterone in androgen biosynthesis in the bovine, and (b) the fecal excretion of the C_{19} androgen metabolites of these C_{21} hormones.

EXPERIMENTAL PROCEDURE

One gram of pregnenolone was dissolved in ethanol and injected into the jugular vein of a Holstein cow, and 1 g. of pregnenolone in olive oil was given subcutaneously, daily, for three successive days to a Holstein bull. An ethanolic solution of 1 g. of 17α-hydroxyprogesterone was injected into the jugular vein of a Holstein cow.

Fecal samples were collected before, during, and after hormone injection, dried in a Freas oven at 45° C., and pulverized in a ball-mill. The dried feces from each collection period were then mixed and 1,500-g. portions bioassayed for androgenic activity by the chick-feeding method previously described (24). Total weight of feces excreted from day to day did not vary appreciably in a given animal. The androgen content of each fecal sample was determined by measuring comb responses of White Plymouth Rock cockerels to the fecal sample incorporated as 10% of the control ration. Fecal samples were substituted for an equivalent amount of alfalfa meal present in the ration. Comb response was expressed as 100 times the ratio of comb weight in milligrams to body weight in grams.

RESULTS

Pregnenolone was injected into a male and a female bovine. Fecal samples were collected before and after hormone administration, and the androgen content of these samples was determined by the chick comb response test. The administration of pregnenolone resulted in a significant increase in the fecal androgens, Table 1. Following the injection of 17α-hydroxyprogesterone into a female bovine, a significant increase in the fecal androgens was noted also (Table 2).

The results indicate that the above 21 carbon atom steroids are metabolized to androgens (C-19 steroids), and demonstrate further the importance of the feces as a pathway for the elimination of steroid hormone metabolites in the bovine.

TABLE 1

Biological estimation of the fecal androgens in the bovine following the administration of pregnenolone

Animal	Pregnenolene treatment	Bovine feces: Collections (days)	Assay chicks: No. of cockerels	Av. body wt.	Av. comb wt.	Comb ratio ± S.E.	Difference after injection
				(g.)	(mg.)		(%)
Holstein bull	Before injection	1	18	299.4	141.3	47.2 ± 3.2	....
	After injection	0–1	18	303.2	179.1	58.4 ± 4.2	+23
		1–2	17	300.5	214.0	70.7 ± 4.5	+50
		2–3	20	302.3	206.1	67.9 ± 4.8	+43
		3–4	17	300.1	197.3	66.6 ± 5.4	+41
		4–5	20	273.2	170.6	62.2 ± 3.7	+31
Holstein cow	Before injection	2	10	172.0	135.6	78.8 ± 6.3	....
	After injection	0–1	16	190.1	223.9	110.2 ± 12.6	+39
		2–3	14	217.6	236.3	106.7 ± 9.2	+35
		3–4	12	176.8	137.2	74.9 ± 8.5	− 5

TABLE 2

Biological estimations of the fecal androgens in the bovine following the administration of 17α-hydroxyprogesterone

Animal	17α-Hydroxyprogesterone treatment	Bovine feces: Collections (days)	Assay chicks: No. of cockerels	Av. body wt.	Av. comb wt.	Comb ratio ± S.E.	Difference after injection
				(g.)	(mg.)		(%)
Holstein cow	Before injection	1	13	249.1	138.0	55.4 ± 7.1	....
	After injection	0–1	18	289.3	168.4	58.3 ± 5.0	+ 5
		1–2	20	252.1	208.3	82.6 ± 9.8	+49
		2–3	17	280.4	233.0	83.1 ± 6.8	+50
		3–4	18	258.7	117.3	45.4 ± 5.2	− 1

DISCUSSION

Previous reports by the authors (11, 13) have demonstrated (a) the biosynthetic transformation of progesterone to fecal androgens in the male and female bovine, and (b) the isolation of androstenedione, together with certain of its transformation products, from bovine feces following the administration of progesterone to a pregnant cow.

The present study reports the biosynthetic transformation of pregnenolone and 17α-hydroxyprogesterone to fecal androgens in the bovine. These results indicate the role of pregnenolone, progesterone, and 17α-hydroxyprogesterone as biochemical precursors of the fecal androgens, and demonstrate the importance of the feces as a pathway for the elimination of the C_{19} androgen metabolites of these C_{21} hormones.

Pregnenolone is regarded as the immediate metabolic precursor of progesterone, and 17α-hydroxyprogesterone is firmly established as the key intermediate in the conversion of progesterone to C_{19} androgens.

The cumulative studies of androgenesis in the bovine provide in vivo results indicating that androgn biosynthesis in the bovine as in many other species involves the sequence pregnenolene → progesterone → 17α-hydroxyprogesterone → androstenedione. However, the results do not allow any conclusion to be drawn about the site where this transformation occurred.

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REPRODUCTIVE CAPACITY OF DAIRY BULLS. V. DETECTION OF TESTICULAR DEFICIENCIES AND REQUIREMENTS FOR EXPERIMENTALLY EVALUATING TESTIS FUNCTION FROM SEMEN CHARACTERISTICS[1, 2]

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SUMMARY

Six ejaculates per week using intensive sexual preparation (6×) were collected from eight 2.5-yr.-old Holstein bulls for 5 wk. before unilateral vasectomy. Daily sperm output (DSO) averaged 4.58 billion and was correlated with paired testes weight at slaughter (+0.97). First ejaculates consistently contained a higher sperm concentration and more total sperm than second ejaculates. After unilateral vasectomy, two bulls were placed on sexual rest, three on two ejaculates per week (2×), and three on eight ejaculates per week (8×) for 20 wk. Even with the 50% decrease in potential sperm output resulting from unilateral vasectomy, sperm concentration (1.07 billion/ml) and total sperm per ejaculate (4.99 billion) for the 2× bulls were essentially normal. However, at the higher frequency (8×), these semen characteristics were clearly deficient (0.63 billion/ml and 1.90 billion). Four requirements suggested for detecting deficiencies in testicular function or for estimating daily sperm production on the basis of DSO under experimental conditions are: (a) high frequency of ejaculation with intensive sexual preparation, (b) short, uniform interval between collections, (c) pre-experimental stabilization of extra-gonadal sperm reserves and rate of sperm resorption, and (d) adequate duration of the experimental period. If sexual activity is adequate, these requirements are met best by collection of two ejaculates daily, preceded by intensive sexual preparation, for an eight- to ten-day pre-experimental period followed by ejaculation at the same frequency for a 2- to 8-wk. experimental period.

There is general agreement that semen characteristics are influenced by ejaculation frequency. This suggests that it is usually not advisable to evaluate a bull's reproductive capacity on the basis of only a few ejaculates collected at irregular intervals. As ejaculation frequency increases the number of sperm per ejaculate decreases, but the total number of sperm collected per week increases (3, 7, 8, 13). Any effect of ejaculation frequency on testicular sperm production would have obvious implications in bull management and particularly in experiments designed to study testicular function on the basis of semen characteristics. As with other glands, it is possible that increased demand might stimulate the testes to produce more spermatozoa. Conversely, sexual rest might depress spermatogenesis. Recently, Amann (5) reported that neither the rate of sperm removal by ejaculation at different frequencies nor vasectomy affected testicular sperm production. Thus, it should be possible to develop a collection

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regime whereby the semen characteristics reflect a bull's capacity to produce spermatozoa.

To establish conditions under which the semen characteristics and daily sperm output (DSO) represent a true balance between testicular sperm production, sperm resorption, and changes in epididymal sperm reserves, it appeared desirable to use bulls with a known deficiency of sperm available for ejaculation. Unilaterally vasectomized bulls were used to simulate a 50% deficiency. This paper considers the influence of ejaculation frequency and the interval between ejaculations on the semen characteristics and sperm output of these bulls, with the objective of establishing conditions necessary to detect this 50% deficiency. It was hoped that requirements for detecting deficiencies in sperm production, both for evaluating individual bulls and for determining treatment effects under experimental conditions, could be evolved.

EXPERIMENTAL PROCEDURE

Two successive ejaculates were collected with the artificial vagina each Monday, Wednesday, and Friday (6×) for a 5-wk. pre-experimental period from each of eight randomly selected 29-mo.-old Holstein bulls. Intensive sexual preparation, consisting of one false mount, 2 min. of restraint, and two additional false mounts was used prior to each ejaculation. Other work (8) suggested that this ejaculation frequency combined with intensive sexual preparation was adequate to permit accurate grouping of the bulls following surgery. The bulls were then unilaterally vasectomized (12) and given a 3-wk. recuperation period. Following uneventful recovery, the bulls were assigned to one of three treatment groups, so that each group had a similar average daily sperm output (DSO) during the pre-experimental period. The postoperative treatment groups, of three bulls each, were sexual rest (SR), two ejaculates per week (2×), and eight ejaculates per week (8×). During the 20-wk. experimental period, the SR bulls (including a third bull not collected at 6× and SR for only 11 wk.) were not collected nor used as stimulus animals. These bulls were subsequently used in studies of extra-gonadal sperm reserves (6) and testicular histology (5). To simulate a collection regime used in A.I. associations, one ejaculate was collected on Monday and Friday from bulls on 2×. To insure maximum demands on the reproductive capacity while considering manpower requirements, semen was collected five days per week from bulls on 8×; two successive ejaculates were collected on Monday, Wednesday, and Friday and one ejaculate on Tuesday and Thursday.

To provide some assurance that the 160 ejaculates required from the 8× bulls in 20 wk. could be collected, this group included those bulls which had showed the highest level of sexual activity on 6×. This did not appear to introduce bias, since the correlation between pre-experimental mean DSO and mean reaction time to the first false mount was not significant ($r = +0.14$). Intensive sexual preparation, as outlined above, was used prior to each ejaculation. The following method was used to maintain adequate sexual stimulation. Two stimulus animals (or combinations of stimulus animals), in separate collection stalls, were used alternately to collect the ejaculates on days when two ejaculates

were collected successively. The stimulus situation used for obtaining the first ejaculate on one day was used for the second ejaculate on the next collection day. When a bull failed to mount within 5 min., the stimulus animal was moved back and forth 3 to 4 ft. for the next 5 min. If a mount still was not elicited, the stimulus situation was changed. Reaction times to both the first false mount and to ejaculation were recorded.

All ejaculates were evaluated by determining the volume of semen, spermatozoan concentration, fructose content of the semen (10), percentage of motile sperm, and percentage of unstained or live sperm (11). The total number of sperm per ejaculate and DSO were calculated from these data. The percentage of sperm with protoplasmic droplets and abnormal morphology were determined by counting 100 cells per ejaculate in the stained preparations. Morphological data were obtained for the first and second ejaculates on one day each week for bulls on 6× and 8× and all ejaculates from bulls on 2×. For analyses of semen and behavior data, the values for the first week were excluded to provide an adjustment period. Thus, the pre-experimental values are averages for the last 4 of 5 wk. and those for the experimental period the last 19 of 20 wk.

RESULTS

Semen characteristics and DSO for the three groups of bulls on 6× during the pre-experimental period were similar (Table 1). While on 6×, DSO for bulls subsequently collected at SR, 2×, and 8× after unilateral vasectomy averaged 4.89, 4.46, and 4.49 billion sperm, respectively. DSO for the eight of the nine bulls collected on 6× was 4.58 ± 0.40 billion and was correlated (+0.97) with paired testes weight at slaughter 23 wk. later. Mean fructose concentration on 6× was variable and ranged from 210 to 544 mg/100 ml. It was not significantly correlated with either ejaculate volume (+0.43) or sperm concentration (−0.57). The significant among-bull correlations between mean reaction time to the first false mount and mean fructose concentration (+0.74) and total fructose per ejaculate (+0.86) were unexpected. Bulls which mounted in the shortest period of time had the lowest levels of seminal fructose.

After unilateral vasectomy, the deficiency of sperm available for ejaculation was apparent only in the 8× group. Sperm concentration and total sperm per ejaculate for the 8× bulls averaged 0.63 billion/ml and 1.90 billion and were clearly deficient. However, of particular interest, from a clinical standpoint, were the essentially normal values for the 2× group (1.07 billion/ml and 4.99 billion). Percentages of motile and live sperm were similar in both groups. The DSO at 8× of 2.17 billion was 49% of that for the same bulls when collected at 6× before unilateral vasectomy.

As reported elsewhere (5), histological data for these nine bulls showed that spermatogenesis was not affected by unilateral vasectomy or ejaculation frequency and daily sperm output. Testicular daily sperm production averaged 10.6 billion (4).

Reaction time for the eight bulls on 6× averaged 3.0 min. to the first false mount and 7.1 min. (including 2-min. restraint) to ejaculation (Table 1).

TABLE 1

Reaction times, semen characteristics, and daily sperm output

Bull group	Ejaculation frequency	No. bulls	No. ejac. per bull	Reaction time to		Ejac. vol.	Sperm conc.	Total sperm /ejac.	Motile sperm	Live sperm	Fructose conc.	Daily sperm output
				1st mount	Ejac.[a]							
				(min.)	*(min.)*	*(ml.)*	*(× 10⁶ /ml.)*	*(× 10⁶)*	*(%)*	*(%)*	*(mg/ 100 ml.)*	*(× 10⁶)*
				Pre-experimental intact								
A[b]	6×	2	24	4.0	8.9	4.4	1,298	5,700	71	86	487	4,886
B	6×	3	24	3.4	7.6	3.9	1,309	5,208	71	85	487	4,464
C	6×	3	24	2.0	5.5	3.5	1,464	5,235	68	84	346	4,487
	Av.	8	24	3.0	7.1	3.9	1,364	5,341	70	85	434	4,578
				Experimental—after unilateral vasectomy								
B	2×	3	38	2.3	6.5	4.6	1,066	4,990	69	83	572	1,426
C	8×	3	151	4.5	9.9	3.0	633	1,895	66	81	453	2,166

[a] Includes the 2-min. restraint period.

[b] Bulls in Group A were sexually rested after unilateral vasectomy.

Thus, the average time between the end of the 2-min. restraint period and ejaculation was 2.1 min. This represents the time required for three mounts and suggests that once a bull has mounted initially, little additional time is expended in the false mounting procedure. One criterion used in assigning the experimental treatments was reaction time on 6×. The bulls with the lowest levels of sexual activity were placed on SR and the more active bulls on 2× or 8×. The methods used to provide adequate sexual stimulation resulted in a relatively low reaction time to ejaculation, which averaged 9.9 min. during the last 19 wk. on 8×. Although a direct comparison was not possible, it appeared that unilateral vasectomy did not affect reaction time.

The percentages of morphologically abnormal sperm were uniformly low in all treatment groups (Table 2). Only two bulls had more than 10% abnormal sperm (both before and after unilateral vasectomy). Even on 6× and 8× the incidence of protoplasmic droplets was only 2%.

Analyses of variance were calculated for data from the first and second ejaculates collected in succession on each of three days per week during the last 4 of the 5 wk. on 6×. These data also included values for three intact mature bulls collected on 6× as outlined. Using intensive sexual preparation for each ejaculation, sperm concentration and total number of sperm per ejaculate were higher ($p < 0.01$) in first than in second ejaculates (Table 3). Volume as well as sperm concentration and total sperm per ejaculate were significantly less for Friday than for Monday or Wednesday collections. Although second ejaculates tended to contain a higher fructose concentration, the difference was significant only on Mondays. The collection days × ejaculates interaction was significant for reaction times to the first false mount and to ejaculation. The second ejaculate on Friday required more time than the first, whereas on Monday and Wednesday the difference was not significant.

DISCUSSION

Although based on limited numbers of animals, the semen data suggest important factors to be considered in evaluation of bulls and in selection of

TABLE 2

Morphology of ejaculated spermatozoa

Bull group	Ejaculation frequency	Abnormal head	Abnormal midpiece	Tailless head	Bent or broken tail	Coiled tail	Droplets		Normal sperm
							Proximal	Distal	
Pre-experimental intact									
A[a]	6×	2.3	1.0	0.5	0.9	0.1	0.5	0.8	94.3
B	6×	3.1	1.5	0.7	1.4	0.2	1.0	0.6	91.5
C	6×	2.0	1.1	0.8	2.6	0.2	0.1	0.4	92.8
	Av.	2.5	1.2	0.7	1.7	0.1	0.5	0.5	92.7
Experimental—after unilateral vasectomy									
B	2×	3.7	1.3	0.8	1.3	0.4	1.0	1.0	90.3
C	8×	2.5	1.3	1.1	1.8	0.3	0.5	1.7	90.9

[a] Bulls in Group A were sexually rested after unilateral vasectomy.

experimental designs. The 50% decrease in potential DSO resulting from unilateral vasectomy simulates a 50% reduction in sperm production. However, with this technique, 100% of the output of one testis is eliminated rather than reducing production of each testis by 50%. Even after unilateral vasectomy, sperm concentration and total number of sperm per ejaculate for the bulls on a low ejaculation frequency (2×) were within the normal range for 30- to 36-mo.-old bulls. However, on the high frequency of 8× there were obvious deficiencies in these characteristics associated with unilateral vasectomy. This demonstrates that at low ejaculation frequencies differences of 50% or less in

TABLE 3

Comparison of first and second ejaculates from 11 bulls collected six times weekly with intensive sexual preparation

Characteristic	1st ejac.	2d ejac.	Diff.
Total No. ejac. collected	130	131	
Ejaculate volume *(ml.)*	4.19	4.15	−0.04
No. sperm per ml. (× 10^9)	1.43	1.03	−0.40[a]
No. sperm per ejaculate (× 10^9)	5.95	4.16	−1.79[a]
Fructose conc. *(mg/100 ml.)*	420	486	+66
Motile sperm *(%)*	68.9	70.1	+1.2
Unstained (live) sperm *(%)*	82.2	84.5	+2.3
Morphologically normal sperm *(%)*[b]	91.7	90.6	−1.1
Reaction time to 1st false mount *(min.)*	2.7	3.4	+0.7
Reaction time to ejaculation *(min.)*[c]	6.5	7.6	+1.1

[a] Significant at the 1% level.
[b] Average for 44 ejaculates per group.
[c] Includes the standard 2-min. restraint period and time required for three false mounts.

semen characteristics between two treatments may not be detected. It is suggested that collection approximating daily ejaculations with intensive sexual preparation for 4 to 8 wk. should be used for detecting deficiencies in a bull's testicular function. This applies even when severe deficiencies are anticipated.

The cyclic pattern in the total number of sperm in two ejaculates per collection day on 6× is shown in Figure 1. After initial adjustment of the extra-gonadal sperm reserves (6) and leveling off of sperm output the first week, the values were relatively constant, with the exception of Fridays. Significantly fewer sperm were collected on Friday. This reduction probably resulted from cyclic partial depletion of the extra-gonadal sperm reserves brought about by collections on Monday and Wednesday. This phenomenon also was observed for the three bulls on 8× and for five nonexperimental, intact mature bulls collected at 6× with intensive sexual preparation prior to ejaculation. These data suggest that there was a build-up of the extra-gonadal sperm reserves from a partially depleted state during the 72-hr. interval between Friday and Monday collections. It has been shown that collection at 6× to 8× results in a 25% reduction of the combined number of sperm in the cauda epididymis and ductus deferens just prior to ejaculation (1, 6). Partial

restoration of the sperm reserves apparently results in an increased rate of sperm resorption in the excurrent ducts (6). Nevertheless, the increase of epididymal reserves over the weekend still was sufficient to permit collection of more sperm on Monday and Wednesday than on Friday. Therefore, it appears that a more uniform collection schedule than 6× would be advantageous.

Daily collections (7×) would fulfill the requirement of minimizing fluctuations in the equilibrium level of the extra-gonadal sperm reserves. With intensive sexual preparation, DSO on 7× is almost as great as on 14× (2, 8). However, it must be concluded that 14× collection (twice daily) is the frequency

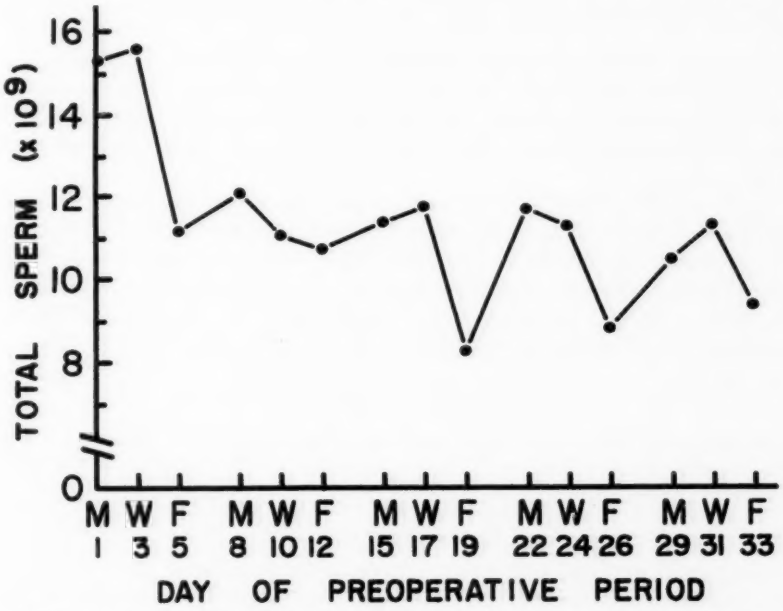

FIG. 1. Average sperm output in two ejaculates for eight 29-mo.-old Holstein bulls during collection at six ejaculates per week, with three false mounts prior to each ejaculation.

of choice to insure detection of small deficiencies in testicular function. In addition to reducing cyclic variations in epididymal reserves, the collection of two ejaculates daily circumvents variations during collection which might affect results obtained on 7×. However, if a bull's level of sexual activity is a limiting factor, once-daily collection should provide relatively accurate data. In any case, intensive sexual preparation should be used. Trials such as this can be used prior to purchasing a bull to reveal any serious deficiencies in sperm output or sexual behavior.

Hale and Almquist (8) suggested the use of a pre-experimental depletion followed by collection at a high frequency such as 7× to evaluate testicular

function. However, because of changes in epididymal sperm reserves following a depletion trial (1), it appears that the best procedure is to allow a preliminary stabilization of extra-gonadal sperm reserves by collecting at the experimental frequency for eight to ten days before recording experimental DSO data. In long-term experiments, periodic semen collections at a high frequency for 2 to 8 wk. following stabilization of epididymal sperm reserves (eight to ten days of collection) should permit detection of differences in testicular function without placing excessive demands on sexual activity. This technique should be valuable in experiments involving variables such as level of exogenous hormone and in assessing effects of dietary and environmental factors. The duration of each experimental collection period depends upon the degree of accuracy desired.

Spermatogenesis requires about 60 days in bulls (4) and sperm can remain in the epididymis for many weeks following isolation from the testis (6, 9). Thus, long experimental periods may be required for sperm output to reflect changes in sperm production. This is true even when an all-or-none treatment response is expected.

Certain features should be considered when attempting to detect testicular deficiencies or when determining the influence of experimental variables on sperm production as evaluated by DSO. These are: (a) high frequency of ejaculation with intensive sexual preparation, (b) short, uniform interval between collections, (c) pre-experimental stabilization of extra-gonadal sperm reserves and rate of sperm resorption, and (d) adequate duration of the experimental period.

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COMPARISON OF QUALITY OF BULL SEMEN COLLECTED IN THE ARTIFICIAL VAGINA AND BY ELECTROEJACULATION[1, 2]

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SUMMARY

Electroejaculated (EE) semen is at least comparable in quality to semen collected in the artificial vagina (AV). The volume of AV semen was less (2.8 vs. 6.3 ml.) and the concentration greater (625 vs. 299 million sperm/ml) than that obtained by EE; however, if sufficient volume of semen is obtained by EE, essentially the same number of total sperm per ejaculate can be obtained by each method. Ninety-four per cent of the EE samples and 68% of the AV samples had an initial motility considered acceptable for artificial insemination. At the end of four days of storage, 55 and 18%, respectively, still had acceptable motility. Samples with a higher pH are usually of inferior quality when compared to other samples collected by the same method, but they are not necessarily inferior to samples collected by a different method. The mean pH for the EE samples (7.3) was 0.6 of a unit higher than for the AV samples, but the EE samples maintained higher motility during four days of storage than those collected in the AV. Since a higher pH and lower concentration of fructose was obtained in the EE samples, it is postulated that the larger volume of fluid comes from the urethral glands, with a slight increase from the seminal vesicles. Semen was obtained more consistently by the EE technique and it is adaptable to nearly all bulls, with no selection on ability or desire to serve the AV.

The artificial vagina (AV) method has become the generally accepted method for collecting semen from bulls. However, some bulls refuse to serve the AV and others, due to advancing age or other reasons, develop physical handicaps which curtail their service. As a result the electroejaculation (EE) method has been developed (2, 9, 10, 12). Several workers (2, 6, 8) have reported that nonreturn rates between semen samples collected by the two methods did not differ. Hill *et al.* (6) reported the volume of samples collected by EE to be 65% of that obtained in the AV, and the pH of EE semen to be approximately 0.4 unit greater. However, no extensive comparison of bull semen collected by these two methods has been reported. This experiment was designed to make such a comparison.

EXPERIMENTAL PROCEDURE

Twelve Hereford bulls, 2 to 6 yr. of age, were selected from a group of 33 bulls, only on the basis of willingness to serve the AV during a preliminary

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period. They were maintained as a single group on hay and corn silage, with access to pasture during the latter part of the experimental period.

An attempt was made to obtain two consecutive ejaculates from each bull on Monday and Friday each week. To compensate for seasonal and other uncontrolled environmental trends, a single crossover design was used. The bulls were divided into two comparable groups of six animals (A and B) on the basis of the average number of sperm obtained per ejaculate during a pre-experimental period.

During the first phase of the experimental period (8 wk.), both first and second ejaculates from each bull were taken by the same method of collection, with the second ejaculate following the first as soon as possible (3-20 min.). Semen was collected from the bulls in Group A for the first 4 wk. in the AV and the next four by EE. During the second phase (4 wk.), the first ejaculate was taken by one method and the second ejaculate approximately 1 hr. later by the alternate method. During the first 2 wk. of the second phase, the first ejaculate from the bulls in Group A was collected in the AV and the second by EE. During the last 2 wk., the first collection was by EE and the second by AV. Throughout the experiment the semen was collected from the bulls in Group B in the same collection sequence but by the alternate method from that used with Group A.

The generally accepted method and equipment were employed in collecting semen samples with the AV. The finger electrode method as described by Parish and Murphree (10) was used to collect the EE semen. An attempt was made to obtain a 6-ml. sample each time (presperm fluid discarded) by this method. The bulls were not teased before collection by either method nor were they intentionally allowed false mounts before serving the AV. The equipment used to collect the semen for each method was warmed and insulated to prevent the sperm from being cold-shocked. A clear plastic jacket was used to insulate the collection vial for the EE method, to allow the operator to observe and control the volume of semen obtained.

Immediately after the semen was collected, the volume was recorded and initial sperm motility in undiluted semen microscopically evaluated. Per cent motile sperm was estimated from 0-100 in increments of ten and the rate of progressive motility evaluated through a range of zero to four. The pH of all samples of sufficient volume (1.5 ml.) was determined, using a Beckman pH Meter (Model G).

Sperm concentration was determined from optical density readings with a Klett-Summerson colorimeter. To convert optical density to sperm concentration, linear regression lines were constructed by comparing the optical density of a 1:39 dilution of semen in 2.8% sodium citrate solution with counts made in a hemocytometer. The correlation coefficients between the two variables were 0.951 and 0.954 for the semen collected by the AV and EE methods, respectively.

Initial fructose concentration of all ejaculates obtained on Friday during Weeks 3-7 and 9-12 was determined by the resorcinol reaction (11), as modified by Erb *et al.* (3).

Maintenance of sperm motility during a four-day storage period for all samples obtained on Monday during Weeks 3 and 4, 7 and 8, and 9 through 12 was evaluated. Within 3 min. after completing each collection, 0.5 ml. of semen was diluted in 4.5 ml. of diluent composed of one part egg yolk to three parts of 3.2% sodium citrate plus 1,000 units of penicillin per milliliter. Two to four hours after collection all samples were further diluted to 12 million total sperm per milliliter by combining aliquots of the previously diluted samples and more of the same diluent. Motility ratings in the diluted semen were made independently by two individuals immediately after the final dilution and after two and four days of storage at 5.0° C.

Statistical analysis of the data for the various criteria was by a weighted "t"-test or Chi-square.

RESULTS

The total number of samples collected by each method would have been 288 if all bulls had ejaculated as scheduled; however, 248 (86%) and 288 (100%) samples were obtained by the AV and EE methods, respectively. Three bulls were responsible for 27 of the 40 failures to ejaculate and one of these bulls refused to serve 15 times. When consecutive samples were obtained by the same method, the interval between EE samples was about 3 min., as compared to 5-20 min. between ejaculates in the AV.

The data on volume, sperm concentration, total sperm, motility, and pH for the entire 12-wk. period are summarized in Table 1. A larger volume of

TABLE 1

Characteristics of semen collected in an artificial vagina or by electroejaculation

Criteria	No. of ejac.[a]		Method of collection	
	AV	EE	AV	EE
Volume *(ml.)*	247	287	2.8	6.3***
Sperm conc. ($\times 10^6$ ml.)	247	287	625	293**
Sperm per ejac. ($\times 10^6$)	247	287	1,693	1,826
Motility[b]	247	287	2.2	2.7**
pH	196	287	6.7	7.3***

** Means differ significantly at the 0.01 level of probability.
*** Means differ significantly at the 0.001 level of probability.
[a] One sample from each method was lost before analyses were completed.
[b] Motility is expressed as the rate of progressive forward motion on a gradient of 0-4.

semen and lower sperm concentration was consistently obtained by EE ($P < .001$). Total sperm obtained by the two methods, however, was not statistically different ($P > 0.5$). The EE semen exhibited a higher rate of progressive motility ($P < 0.01$) and also had a higher pH ($P < C.001$).

A summary of the data on total number of sperm obtained in the first and second ejaculates during the first and second phases is presented in Table 2. During the first phase, when consecutive ejaculates from each bull were collected by the same method, there was no statistical difference between ejaculates or methods in the number of sperm obtained. During the second phase, when the first ejaculate was obtained by EE and the second by AV, significantly less sperm ($P < .001$) were obtained in the second ejaculate.

TABLE 2

Effect of collection method sequence on total number of sperm per ejaculate

Sequence	No. ejac.	Mean No.[a] sperm ($\times 10^6$)
First phase		
1st ejaculate in AV	91[b]	1,818
2nd ejaculate in AV	80	1,593
1st ejaculate by EE	96	1,758
2nd ejaculate by EE	96	1,304
Second phase		
1st ejaculate in AV	42	2,092
2nd ejaculate by EE	47[b]	2,078
1st ejaculate by EE	48	2,368
2nd ejaculate in AV	34	1,057[c]

[a] Weighted mean of all observations based upon the number of observations for the artificial vagina method of collection for each bull.

[b] One sample of semen collected by each method was lost before being evaluated.

[c] The second artificial vagina samples, when preceded an hour before by electroejaculation, had significantly ($P < .001$) less sperm than the second electroejaculated samples.

The initial fructose concentration was 3.3 (EE) and 5.5 (AV) mg. per milliliter. The EE samples, however, contained more total fructose (20.3 mg. EE, 15.4 mg. AV) and 2.1 mg. more fructose per billion sperm.

In comparing the motility ratings of all stored samples, the EE samples consistently had a higher motility rating during the four-day storage period; however, these differences were not significant. At the beginning of storage, 94 and 68%, respectively, of the EE and AV samples were considered to be acceptable for storage to use in artificial insemination (i.e., rate and per cent motility of at least two and 50, respectively). At the end of four days of storage, 55% of the EE and 18% of the AV samples still had a motility rating acceptable for artificial insemination (Table 3). This difference was significant ($P < .02$).

TABLE 3

Motility ratings by samples collected in the artificial vagina and by electroejaculation stored for a four-day period at 5° C.

		AV Storage interval		EE Storage interval	
	Days	0	4	0	4
Mean motility rating for all samples stored	No. sample	79	79	96	96
	Degree[a]	2.5	1.3	2.8	1.9
	Per cent[b]	52	34	65	53
Acceptable samples (2-50)[c]	No. sample	54	14	90	53[d]
	Degree	2.9	2.1	2.9	2.3
	Per cent	64	60	67	64

[a] Degree of progressive motility (0-4).

[b] Per cent of motile sperm (0-100).

[c] To be acceptable, a sample had to have a degree of progressive motility rating of at least 2, and 50% or more motile sperm.

[d] Significantly ($P < .02$) more electroejaculated than artificial-vagina samples were still acceptable at the fourth day of storage.

DISCUSSION

Only two of the 12 bulls used in this study showed any indication of loss of libido following EE collections. These two bulls failed only one time each to give a second ejaculate when collecting both ejaculates with the AV during the first 8 wk.; however, during the second phase, when the first ejaculate was taken by EE, no second ejaculates were collected in the AV. With the exception of these two bulls there was no evidence of a detrimental effect on any bull's libido within 1 hr. after electroejaculation. Considering these facts, and the consistency with which the other bulls worked, this experiment confirms other reports (2, 9) that bulls do not form any unfavorable associations with electrical stimulation.

The marked reduction in total sperm collected in the AV samples, when preceded within an hour by EE, suggests temporary impairment of a bull's ability to ejaculate naturally. The inhibiting effect is, however, of short duration, since three or four days after the EE collections each bull produced a normal first ejaculate in the AV.

Since the volume of semen obtained by EE was standardized to 6 ml. in this investigation, the variation in concentration was responsible for almost all of the variation in total sperm ($r = .97$). In the AV samples, however, variation in total sperm was about equally a result of the variation in volume ($r = .65$) and concentration ($r = .72$). These two factors seem to contribute independently to the increase in total sperm, since they are negatively correlated with each other.

The more concentrated samples, and those with the greatest number of total sperm, when collected by either method, stored better on the average than samples less concentrated or containing less total sperm. The number of AV samples initially acceptable for storage and the number storing successfully is lower than would be expected from bulls used in routine artificial insemination. Contributing factors may have been that these bulls were not selected for semen quality, were not teased prior to collection, and the smaller AV samples may have been more susceptible to cold shock and damage by contamination. However, it must be noted that while these samples failed to maintain acceptable motility, over 50% of samples collected from the same bulls by EE and handled under the same conditions after collection were considered acceptable for insemination after four days of storage.

Lutwak-Mann and Rowson (7) reported that fructose concentration in the presperm fluid varied from 0.02 to 0.37 mg. per milliliter and that it increased greatly in the sperm-containing fraction (0.84 to 6.50 mg. per milliliter); pH of the presperm fluid varied from 7.8 to 8.0 as compared to 6.7 to 7.0 in the sperm-containing fractions. Hess *et al.* (5) reported that, after removing the seminal vesicles, the mean semen fructose concentration of four bulls dropped from 2.56 to 0.10 mg. per milliliter and the pH increased from 6.6 to 7.8. These data indicate that the seminal vesicles secrete the major portion of the fructose in bull semen. In the present investigation, there was a negative correlation between pH and fructose concentration in the semen collected by both methods. These results and the results of others (5, 7) indicate that the high pH fluid

comes from glands that do not secrete fructose; the major contribution probably is by the urethral glands.

The high pH fluids secreted by the bulls in this study reduced slightly the rate of sperm movement but did not affect the per cent of motile sperm. These data support the findings of others (1, 4), that a high pH adversely affects semen quality. This is not true, however, when comparing the semen obtained by one method of collection with the semen collected by another method. Within each method of collection the samples with the higher pH values were of lower quality; but, even though the EE samples had a significantly higher pH (7.3 vs. 6.7), on the average, they were not of inferior quality as compared to the AV samples. This is borne out by the fact that significantly more EE samples had an acceptable initial motility and maintained a higher rate of motility ($P < .02$) through four days of storage (Table 3).

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LIPID PHOSPHORUS AND NUCLEIC ACID PHOSPHORUS CONTENT OF BULL SEMEN DURING AERATED AND NONAERATED INCUBATION[1, 2]

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SUMMARY

Seventeen samples of semen from six bulls had an average lipid phosphorus (P_{lipid}) content of 0.068 mg. per milliliter and an average nucleic acid phosphorus ($P_{nucleic\ acid}$) content of 0.132 mg. per milliliter. Incubation of these samples as a thin layer in Erlenmeyer flasks (aerated) or in culture tubes (nonaerated) failed to decrease significantly P_{lipid} or $P_{nucleic\ acid}$ or to increase total acid-soluble phosphorus (P_{TAS}). Inorganic phosphorus (P_I) increased with both aerated and nonaerated incubation, as previously reported with nonaerated incubation. Although the source of the increase was not established and apparently did not orginate from P_{lipid} or $P_{nucleic\ acid}$, there is no conflict, at least in nonaerated incubation, with the hypothesis of ATP breakdown.

A concept of Lardy and Phillips (10), that the lipid phosphorus (P_{lipid}) content of bull semen and of washed sperm substantially decreases during storage, has not been supported in work with washed sperm of the bull (3) or of the ram (9). With washed ram sperm aerobically incubated at 37° C. up to 6 hr., Hartree and Mann (9) noted only a slight decrease in P_{lipid} or in plasmalogen phosphorus ($P_{plasmalogen}$). Plasmalogen is the chief lipid in the semen of the ram, the bull, and several other species. Bomstein and Steberl (3) did note some decrease in P_{lipid} with once-washed, but not twice-washed, bull sperm.

According to Lardy and Phillips (10), total acid soluble phosphorus (P_{TAS}) increased during 56-hr. storage of diluted bull semen at 10° C., to partially account for their decrease in P_{lipid}. Ehlers *et al.* (5), however, noted no significant increase in P_{TAS} during 3 hr., 37° C. incubation of bull semen.

Fatty acids were freed from lipids of ram sperm, although without liberation of phosphate, during both anaerobic and aerobic incubations by Hartree and Mann (9). These fatty acids were suggested as substrates for endogenous respiration, and in this sense do not contradict the report (10) that lipids are oxidizable sperm reserves.

Although Hartree and Mann (9) found the bulk of plasmalogen in sperm, some was always present in the plasma of ram semen. They observed that plasmalogen decreased in sperm and increased in seminal plasma as a result of cold shock. Blackshaw and Salisbury (2) similarly reported that cold shock of bull semen resulted in passage of P_{lipid} from sperm to plasma.

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Salisbury *et al.* (13) noted a marked decrease of desoxyribose nucleic acid (DNA) in sperm during mildly aerobic 5° C. storage of bull semen in yolk-citrate. These findings were based on the Feulgen reaction and microspectroscopy. Presumably, decrease in either ribose or desoxyribose would be detected also by measurement of nucleic acid phosphorus ($P_{\text{nucleic acid}}$).

The principal purpose of the present study was to observe possible decreases in P_{lipid} and $P_{\text{nucleic acid}}$ which might account for the increase in P_I upon incubation of bull semen. Except for one trial using whole semen (10), previous work on P_{lipid} changes with incubation have been with washed sperm (3, 9, 10). The reactions which might bring about an increase in P_I from P_{lipid} or $P_{\text{nucleic acid}}$ would probably be oxidative in nature. Minimal and maximal aeration was suggested, however, because the P_I increase previously reported (5, 8) had been with minimal aeration.

MATERIALS AND METHODS

Semen diluted with four parts of 2.9% sodium citrate was sampled for phosphorus fractions, fructose, and lactic acid at 0 hr., divided into aliquots designated aerated and nonaerated, and again sampled after 3 hr. at 37° C. Aeration consisted of incubation of the semen-citrate as a thin layer within Erlenmeyer flasks and giving a liquid surface to volume ratio of 4.71 $\left(\frac{\text{S in sq. cm.}}{\text{V in ml.}} = 4.71\right)$. Nonaerated incubation of semen-citrate, within culture tubes, had a corresponding 0.13 ratio. At each of the three samplings, 3 ml. of semen-citrate was deproteinized with 12 ml. of cold 10% trichloroacetic acid. After centrifugation and filtration, the following semen equivalent quantities (milliliters) of the filtrate were used for these determinations: fructose, 0.01; lactic acid, 0.02; P_I, 0.02; P_{I+H}, 0.08; (P_{I+H} = inorganic + easily hydrolyzable phosphorus); and P_{TAS}, 0.016. $P_{\text{nucleic acid}}$ was determined on 0.04 ml. of semen equivalent within the trichloroacetic acid precipitate. P_{lipid} and P_{Tot} (total phosphorus) were calculated on 0.06 and 0.016 ml. of semen equivalent in separate samplings of the semen-citrate mixture. These procedures were not designed to completely partition phosphorus in semen.

The fructose method was a modification (6) of Roe (12) and the lactic acid method was that of Barker and Summerson (1). Phosphorus was estimated by the method of Fiske and Subbarow (7) after suitable extraction procedures. P_I, P_{I+H}, P_{TAS}, and P_{Tot} extractions were as previously described (5). P_{lipid} was extracted by an ethanol-ether method (16) and $P_{\text{nucleic acid}}$ extracted according to Schneider (14). P_{lipid} and $P_{\text{nucleic acid}}$ fractions were then wet-washed using concentrated sulfuric and 70% perchloric acid similar to that previously described for P_{TAS} and P_{Tot} (5).

Because of evaporation during the 3-hr. incubation, particularly from the Erlenmeyer flasks, the 3-hr. aerated and nonaerated values of constituents were adjusted to compensate for fluid loss. This loss was determined by the difference between P_{Tot} before and after incubation and amounted to 8.3% in the aerated and 0.9% in the nonaerated incubations.

Sperm concentration was estimated with hemocytometer and motility with low-power microscopy. Statistical analysis was according to Snedecor (15).

RESULTS AND DISCUSSION

Seventeen semen samples from six bulls provided the data of Table 1. The P_{lipid} value of 0.068 mg. per milliliter of semen, with 95% confidence limits of 0.059 to 0.077, compares with a previously reported 0.09 mg. mean on ejaculates of ten bulls (11) and 0.276 and 0.38 mg. (10) on single ejaculates. A P_{lipid} value of 0.072 mg. per 10^9 bull sperm was calculated from tabular data on 13 samples reported by Bomstein and Steberl (3). Hartree and Mann (9) noted a $P_{plasmalogen}$ content of 0.058 mg. in one sample of bull semen. Apparently, no data are available to compare with the 0.132 mg. of $P_{nucleic\ acid}$ in Table 1.

Calculation of 95% confidence limits on P_I, P_{I+H}, P_{TAS}, and P_{Tot} of Table 1 showed that these limits encompassed the means of a previous report by this laboratory (5). Comparisons with other P_I, P_{TAS}, and P_{Tot} findings can be noted in this previous study (5).

The failure of P_{lipid} and $P_{nucleic\ acid}$ to decrease significantly and of P_{TAS} to increase significantly (Table 1) indicates that P_{lipid} and $P_{nucleic\ acid}$ do not account for the increase in P_I with either aerated or nonaerated incubation of semen. Although the present incubating conditions may not have been most conducive to P_{lipid} alterations, because of low oxygen potential within the nonaerated samples and the delatively high available carbohydrate within the aerated samples, the findings are not in harmony with the previously reported pronounced decrease in P_{lipid} and the increase in P_{TAS} upon storage of diluted whole semen (10). The data give no indication that P_{lipid} decreases in the presence of seminal plasma, as may be suggested by the decrease in P_{lipid} of once-washed, but not twice-washed sperm, reported by Bomstein and Steberl (3).

P_I and P_{I+H} increased during both aerated and nonaerated semen incubation (Table 1), as has been noted for nonaerated incubation (5). No explanation is offered as to the apparent differences between P_I and P_{I+H} increases with aerated and nonaerated incubation (Table 1). The greater increase in P_I than in P_{I+H}, in the nonaerated incubation, is compatible with the suggestion (5) that increase in P_I is due partially to irreversible breakdown of ATP.

Fructose utilization (0 minus 3 hr. fructose) and lactic acid accumulation (3 minus 0 hr. lactic acid), as might be expected, were considerably lower with aerated incubation, being about one-half that of nonaerated. This may be related to the differences in phosphorus metabolism, although certainly the reason is not clear. The lower rate of fructolysis in the aerated samples failed to give a higher mean percentage of sperm motility after 3 hr. of incubation (Table 1). This result supports the generally accepted concept favoring anaerobic storage of sperm. These similar mean motilities were noted, even though six samples of nonaerated semen had reached an extremely critical stage of fructose supply (< 0.25 mg. per milliliter incubate) as compared with one aerated sample.

TABLE 1

Phosphorus, fructose, lactic acid, sperm concentration, and sperm motility in 17 samples of semen (six bulls) incubated 3 hr. at 37° C. in four parts sodium citrate buffer under aerated and nonaerated conditions

Measurement		After incubation at 37° C.			Remarks [a]
		Preincubation 0 hr.	3 hr. aerated	3 hr. nonaerated	
		(mg. per ml. semen)			
P_{lipid} (lipid phosphorus)	Mean	0.068	0.070	0.069	No significant change in P_{lipid} with aerated or nonaerated incubation.
	Std. dev.	0.018	0.020	0.021	
$P_{nucleic\ acid}$ (nucleic acid phosphorus)	Mean	0.132	0.136	0.125	No significant change in $P_{nucleic\ acid}$ with aerated or nonaerated incubation.
	Std. dev.	0.036	0.036	0.034	
Acid-soluble phosphorus					
P_I (inorganic phosphorus)	Mean	0.037	0.041	0.046	Increase in P_I ($P < 0.1$) with aerated incubation.
	Std. dev.	0.017	0.013	0.018	Increase in P_I ($P < 0.01$) with nonaerated incubation. Greater increase in P_I ($P < 0.01$) with nonaerated than aerated incubation.
P_{I+H} (inorganic + easily hydrolyzable phosphorus)	Mean	0.056	0.062	0.060	Increase in P_{I+H} ($P < 0.01$) with aerated and nonaerated incubation.
	Std. dev.	0.022	0.024	0.023	Greater increase in P_{I+H} ($P < 0.01$) with aerated than nonaerated incubation.
P_{TAS} (total acid-soluble phosphorus)	Mean	0.495	0.493	0.505	No significant change in P_{TAS} with aerated or nonaerated incubation.
	Std. dev.	0.136	0.137	0.153	
P_{Tot} (total phosphorus)	Mean	0.943	.....	.....	
	Std. dev.	0.260	.....	.....	
Fructose	Mean	6.04	3.80	2.43	Decrease in fructose ($P < 0.005$) with aerated and nonaerated incubation.
	Std. dev.	1.28	1.94	1.79	Greater decrease in fructose ($P < 0.05$) with nonaerated than aerated incubation.
Lactic acid	Mean	0.69	2.37	3.91	Increase in lactic acid ($P < 0.005$) with aerated and nonaerated incubation.
	Std. dev.	0.11	1.18	1.14	Greater increase in lactic acid ($P < 0.01$) with nonaerated than aerated incubation.
	($\times 10^9$ per ml. semen)				
Sperm concentration	Mean	1.12			
	Std. dev.	0.30			
	(per cent progressively motile)				
Sperm motility	Mean	67	20	20	Decrease in motility ($P < 0.005$) with aerated and nonaerated incubation.
	Std. dev.	7	13	16	No significant difference in motility decrease with aerated and nonaerated incubation.

[a] Significance determined by "t" test.

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TECHNICAL NOTES

IODINE LOSSES IN THE ALKALINE ASHING METHOD FOR DETERMINATION OF PLASMA PROTEIN-BOUND IODINE [1, 2]

The alkaline ashing method of Barker *et al.* (2), as originally proposed or as modified in one or more details, has found wide application in the determination of plasma protein-bound iodine (PBI). The method consists of precipitating and washing the plasma proteins, drying and ashing the precipitate with alkali, dissolving the ash with acid, and colorimetric determination of the iodide by its catalytic effect on the arsenious acid reduction of ceric sulfate. In the alkaline ashing procedure, iodine losses ranging from 0 to 25% have been reported by various workers (1, 2, 5, 7). Foss *et al.* (4) have recently made a careful study of the sources of iodine losses in the procedure.

The present report indicates the magnitude of iodine losses in the PBI method of Brown *et al.* (3) (modification of Barker's method), as adapted by Lennon and Mixner (6).

MATERIALS AND METHODS

One estimate of iodine losses in PBI determinations following protein precipitation was obtained by comparing PBI values obtained by the Brown method with those obtained by an isotope dilution procedure in plasma samples from six mature dairy cows. In the isotope dilution method, 0.02 μc. of carrier-free sodium radioiodide (Abbott Laboratories) was added to the precipitated proteins from 1 ml. of plasma. After drying, ashing, and acidification of the ash, the amount of iodine in each sample tube (determined by colorimetry) and the amount of radioactivity (measured with a well-type scintillation counter) were used to calculate the specific activity of iodine. Since the amount of radioiodide added was negligible, the amount of iodine in each tube was determined by simply dividing the total radioactivity added by the final specific activity of iodine.

Iodine losses were also studied by adding 0.1 μc. of I^{131}-labeled L-thyroxine to 8-ml. samples of plasma from six other cows and measuring the recovery of radioactivity after certain steps (precipitation and washing of the plasma proteins, acidification of the ash) in the PBI procedure. Before beginning the PBI procedure, the radiothyroxine was allowed to equilibrate with the plasma for 30 min. The plasma was then passed through an ion exchange column (PBI Rezikit, E. R. Squibb and Sons) to remove any contaminating radioiodide.

RESULTS

Isotope dilution techniques enable accurate measurement of unknown quantities without requiring quantitative recovery of the unknown at the end of a separation procedure. An indication of the ability of the chemical PBI procedure to measure PBI quantitatively was obtained by comparing it with the isotope dilution method (Table 1). The average recovery of PBI in six plasma samples following the protein precipitation step was 96.1%. An analysis of variance indicated that differences in PBI between the two methods and among animals were highly significant ($P < 0.01$).

TABLE 1

Loss of plasma protein-bound iodine following precipitation of plasma proteins

Animal No.	Method		% Recovery by chemical method
	Isotope dilution PBI, μg/tube	Chemical PBI, μg/tube	
H224	.0425[a]	.0416	97.9
H229	.0461	.0445	96.5
H233	.0543	.0524	96.4
H237	.0403	.0384	95.3
H245	.0486	.0457	94.1
H247	.0475	.0457	96.1
Mean	.0466	.0447	96.1

[a] Each value in the table represents a mean of two determinations.

When I^{131}-labeled thyroxine was added to plasma, 99.7% was recovered following protein precipitation and washing (Table 2). After drying, ashing, and acidification of the ash, however, the recovery of radioactivity was down to 96.7%.

Thus, two different procedures indicated that about 4% of the iodine was lost during

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers—The State University, New Brunswick.

[2] This study was part of a Northeast Regional Research Project (NE-41, Endocrine Factors Affecting Reproduction and Lactation in Dairy Cattle), a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds of the United States Department of Agriculture.

TABLE 2

Recoveries of thyroxine-I^{131} at the end of certain steps in the plasma protein-bound iodine procedure

Animal No.	Completed step: Protein precipitation and washing	Completed step: Drying, ashing, and dissolving of ash
	(%)	(%)
H201	99.0[a]	96.8
H971	97.9	96.3
H973	98.8	96.5
H977	100.9	96.4
H984	99.6	96.9
H985	102.2	95.1
Mean	99.7	96.7

[a] Each value represents the mean of two determinations.

the alkaline ashing PBI method. No losses occurred during precipitation and washing of the proteins. As indicated by Foss *et al.* (4), the major losses probably occur during the incineration and acidification of the ash.

SUMMARY

Two different radioisotope procedures were used to determine iodine losses during different steps in the assay for protein-bound iodine in plasma of cattle by the alkaline ashing method. In the first procedure, radioactive iodide was added to the washed precipitated proteins of six blood samples. Losses in iodine between this step and final colorimetry averaged 3.9%. In the second procedure, radioactive thyroxine was added to whole plasma of six cows. Losses in iodine following washing and precipitation of proteins were negligible (0.3%) and were 3.3% for the entire procedure.

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SUBSTRATE SPECIFICITY OF PREGASTRIC ESTERASE FROM THE CALF[1]

The substrate specificity of pregastric esterase from the calf has not been investigated extensively. Harper (3) and Farnham *et al.* (2) reported that extracts of certain oral and pharyngeal tissues (1) hydrolyze the glyceride linkages of milk fat involving butyric acid more rapidly than those of higher fatty acids. Though the tissues used in these studies are believed to contain the glands that secrete pregastric esterase (7), it is probable that esterases from nonsecretory tissue also were involved. Thus, further study on the substrate specificity of pregastric esterase was undertaken.

[1] This investigation was supported in part by a research grant, A-2230, from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

EXPERIMENTAL PROCEDURE

These preparations of pregastric esterase were used:

1. Aqueous extract of oral tissues taken from the base of the tongue and the pharyngeal end of the esophagus, designated as B, C, and D of Figure 1 in a previous report (7).
2. Saliva, aspirated from the base of the tongue.
3. Sham-fed nonfat milk (9).

Though the tissue extract may have contained nonsecretory esterase, in addition to

pregastric esterase, it was believed that the saliva and sham-fed nonfat milk contained only the latter enzyme. In addition to the foregoing preparations, aqueous extracts of the parotid salivary gland, the submaxillary salivary gland, and the pancreas were studied for comparative purposes.

Two types of substrate were employed in this study: esters of 2-naphthol (acetate, butyrate, caprylate, laurate, myristate, and palmitate) and milk fat. The assay technique for 2-naphthyl esters was that of Nachlas and Seligman (5), as modified by Ramsey *et al.* (7). No sodium taurocholate was included in the substrate mixture, however, since this compound reduces the activity of pregastric esterase. The hydrolysis of each ester was followed progressively by stopping the reaction at various time intervals after addition of the enzyme. Total length of incubation ranged from a few minutes to 1 hr., depending on rate of hydrolysis.

The reaction mixture for the assays involving milk fat was as follows: 10 ml. of evaporated milk, 3 ml. of 1.0 *M* phosphate-citrate buffer, and 5 ml. of enzyme solution. When the activity of pancreatic tissue was measured, 50 mg. of sodium taurocholate were included to activate the lipase. The temperature of incubation was 35° C. At various intervals throughout the incubation period, tubes containing the reaction mixture were removed and heated at 85° C. for 15 min. to inactivate the enzyme. Subsequently, the free fatty acids present in 5 ml. of the inactivated samples were separated chromatographically, by modifying a previous technique (9), into two fractions: butyric acid and higher fatty acids. Procedural changes introduced in this study involved use of 20- by 400-mm. chromatographic tubes, in which were packed 60 g. of the mixture of silicic acid and ethylene glycol reagent. The mobile phase was 1% *n*-butanol in hexane, and the effluent was collected either in 5- or 10-ml. fractions on an automatic fraction collector.

RESULTS AND DISCUSSION

The substrate specificities of saliva, the oral tissues, the submaxillary salivary gland, and the parotid salivary gland were examined with the 2-naphthyl esters. For all combinations of enzyme preparations and esters, the rate of hydrolysis decreased progressively with increasing length of incubation. Since this was suggestive of a first-order reaction, an attempt was made to linearize the data (8) by transforming "per cent hydrolysis" to "2 − log (100 − % hydrolysis)." Examination of the transformed data indicated that only the initial reaction rate was first-order in most cases. Accordingly, specificity values were based on estimates of the initial rates of hydrolysis only. Such estimates were obtained by first computing a slope, using the least squares method, from the initial points of transformed data that seemed to lie in a straight line. Subsequently, relative specificity values were computed by expressing the slope for each ester as a percentage of the slope observed for butyrate. The values so obtained are presented in Table 1.

All sources of esterase exhibited a higher specificity for butyrate than for any of the other esters. With the exception of acetate, the rate of hydrolysis decreased with increasing molecular weight of the fatty acid. The patterns for saliva and for the extract of oral tissues were similar and were characterized by values for laurate, myristate, and palmitate that appeared to be higher than those noted for the submaxillary and parotid salivary glands. The parotid extract had a higher relative specificity for acetate than for caprylate, whereas the reverse was true for the other sources of esterase.

The specificity patterns of three enzyme preparations were examined with milk fat: sham-fed nonfat milk, and extracts of the oral tissues and the pancreas. In no case did any reaction appear to be first-order. Thus, the results of these assays are presented simply as titration values (Table 2). During the initial stage of incubation, the hydrolysates containing the sham-fed nonfat milk and the extract of oral tissues exhibited higher concentrations of free butyric acid than of free higher fatty acids. Though not reported in Table 2, similar results were obtained in an assay with sham-fed water. Because milk fat contains only 10.4 *M* % of butyric acid (4), the marked preference of pregastric esterase

TABLE 1

Relative specificity of esterases from various sources for different esters of 2-naphthol [a]

Source of enzyme	No. of observations [b]	Mean relative specificity for different esters					
		C_2	C_4	C_8	C_{12}	C_{14}	C_{16}
Saliva	4	21	100	55	16	3	1.5
Oral tissues	2	19	100	54	8	4	1.8
Submaxillary salivary gland	2	19	100	56	2	0.4	0.2
Parotid salivary gland	2	45	100	16	0.3	0.2	0.1

[a] The pH and temperature of incubation were 6.6 and 37° C., respectively.
[b] Observations within each source of enzyme were made on different animals.

TABLE 2

Specificity of esterases from various sources for butyric and higher fatty acids in milk fat

Source of enzyme	No. of observations	pH of buffer	Length of incubation	Free fatty acids in incubation mixture [a]	
				Butyric	Higher
			(hr.)	*(μM per 5 ml.)*	
Sham-fed nonfat milk	2	6.0	0.25	34	28
			1	60	58
			4	69	89
			12	82	139
Oral tissues	1	6.0	1	37	35
			4	64	75
Pancreas	1	7.8	0.25	10	45
			1	30	110
			4	53	214
			12	74	382
Pancreas	1	7.8	0.67	16	52
			2	29	145

[a] Five milliliters of the incubation mixture contain approximately 990 *μM* of total fatty acids.

for glyceride linkages involving this acid is plainly indicated. To illustrate the degree of specificity further, it was estimated that 59% of the butyrate linkages were hydrolyzed by the pregastric esterase in sham-fed nonfat milk during the first hour of incubation, whereas only 7% of the higher fatty acids were released by this enzyme during that period.

The lipolytic enzymes of the pancreas, on the other hand, appear to be considerably less specific for esters of butyric acid than is pregastric esterase. Throughout the incubation period, the concentration of higher fatty acids always exceeded that of butyric acid. Moreover, the ratio between these two fractions remained fairly constant, with butyric acid averaging 19% of the total free fatty acids. This proportion is somewhat higher than the 8.4% observed by Harper (3). Thus, the observations in this study definitely indicate that pregastric esterase in the calf has a high relative specificity for esters of butyric acid. Moreover, the specificity pattern of this enzyme system appears to be unlike that of the esterases in extracts of the pancreas and of the parotid and submaxillary salivary glands. Though the participation of the foregoing salivary glands in pregastric esterase secretion has not been established, it has been suggested that they probably are not involved (9). The difference noted above in substrate specificity patterns would seem to support this view. A recent investigation, however, suggests that pregastric esterase probably consists of at least two different esterases (6). Thus, if each esterase possessed a different substrate specificity pattern, and if such enzymes were present in various ratios among different tissues, extracts of such tissues, though containing the same esterases, would yield dissimilar specificity patterns.

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SYMPOSIUM: Recent Advances in Reproductive Efficiency[1]

ESTROUS CYCLE AND OVULATION CONTROL IN CATTLE

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The development of artificial methods for regulating the estrous cycle in various classes of farm animals has long been a goal of animal physiologists. Interest in the subject has been intensified in recent years as a result of the discovery of new information regarding factors affecting ovarian function and the estrous cycle, and because of the development of effective oral steroid hormone preparations.

When a practical method for estrous cycle regulation in cattle is finally developed to the point where large numbers of animals can be successfully bred on a single day, it will probably find its best application in commercial beef cattle operations, where it will make artificial insemination a widespread practice. In the dairy industry the method will probably be used mainly to breed groups of heifers on a single day. This will enable more heifers to be bred artificially and will reduce the number bred naturally while on pasture to bulls of questionable genetic merit. The increasing size of dairy herds and the increasing emphasis on greater efficiency of production have combined to encourage the practice of raising large numbers of replacement heifers as a specialized operation. Development of successful methods for estrous cycle regulation may be expected to accelerate this trend, since it will make it possible to market groups of heifers bred to calve on given dates.

Some basic concepts. An appreciation of recent advances made in our knowledge of the mechanisms regulating ovarian function in cattle is essential to a clear understanding of the developments now occurring in the field of estrous cycle regulation.

The basic interactions between the pituitary and the ovary, shown in a somewhat oversimplified form in Figure 1, were worked out and generally accepted prior to 1950.

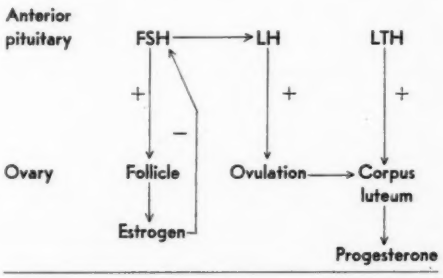

FIG. 1. Interrelations between the anterior pituitary and the ovary.

Follicle growth and ovum maturation occur primarily under the influence of FSH, although small amounts of LH undoubtedly synergize the reaction. This rapid follicular growth, which occurs between the 16th day of the cycle and the beginning of estrus in the cow, results in increased estrogen production, which, in addition to bringing the animal into estrus, is thought to inhibit the pituitary production of FSH and facilitate the release of amounts of pituitary LH sufficient to cause ovulation. The gonadotrophin-producing basophils (delta cells) in the bovine pituitary undergo a very rapid process of degranulation just before the beginning of estrus, which may represent the final FSH release which causes preovulatory swelling of the follicle [Hansel *et al.*, (8)]. LH release in the cow probably occurs at some later time, during the 18-hr. period of estrus (8), and ovulation results, on the average, 11 hr. after the end of estrus.

Following ovulation, the corpus luteum begins to develop and the third pituitary gonadotrophin, LTH, is thought to cause it to secrete progesterone. However, it should be emphasized that it has not yet been possible to specifically demonstrate a luteotrophic hormone in the bovine. The corpus luteum normally increases in size and progesterone content until the 16th day of the cycle, at which time it begins to regress rapidly. Simpson (18) has given an excellent detailed account of these interrelationships between the gonadic and gonadotrophic hormones.

Since 1950 a great deal of information has accumulated to show that the hypothalamus, and higher nerve centers as well, exert marked influences over the secretion of the pituitary gonadotrophins. Results of experiments conducted in this area have given rise to a neurohumoral concept of the mechanism of ovulation and corpus luteum formation. It appears that the release of the pituitary gonadotrophins necessary for follicle maturation, ovulation, and corpus luteum formation is controlled by one or more neurohumors produced in hypothalamic nuclei, or higher nerve centers, and transported to the anterior lobe of the pituitary by way of a specialized hypophyseal portal vascular system. Evidence for this concept, especially as related to the bovine, was reviewed recently in some detail by Hansel (6).

[1] Paper presented at a Joint Production-Extension Symposium at the 56th Annual Meeting of the American Dairy Science Association, University of Wisconsin, Madison, June 14, 1961.

Little is known of the nature of these hypothalamic mediators, or of the exteroceptive pathways to the hypothalamus and the stimuli which activate them in various species. Although incompletely understood, this mechanism is of particular importance, because it changes our concepts of how gonadal hormones influence gonadotrophic hormone secretion and provides a partial answer to the question of how changes in an animal's environment are converted into changes in the nature and quantities of gonadotrophins secreted.

Studies aimed at finding the nature of the hypothalamic neurohumors, and the environmental factors which influence their release in the cow, have provided two new methods for altering the cycle.

The first of these is by daily subcutaneous injections of oxytocin during either the first seven or the third to sixth days of the estrous cycle, inclusive. This treatment prevents the normal formation of the corpus luteum and results in a precocious estrus and ovulation at approximately the eighth to tenth day of the cycle [Armstrong and Hansel (2)]. Ovulation then occurs normally and subsequent cycles are of a normal length.

The second method involves dilatation of the uterus during the first third of the estrous cycle with small, self-retaining rubber catheters held in place by balloons filled with water [Hansel and Wagner (12)]. This treatment also prevents the normal formation of the corpus luteum and results in a precocious estrus and ovulation at the eighth to twelfth day of cycle. These methods of recycling an animal will be discussed further, in a later section.

Earlier studies on estrous cycle synchronization in cattle. This subject was reviewed in detail in 1955 by Ulberg (23), in 1958 by Donker *et al.* (4), and by Hansel (6) in 1959, so that only broad generalizations summarizing our knowledge up to that time need be made here.

It has long been known that removal of the corpus luteum by manipulation of the ovary through the rectal wall results in estrus and ovulation within two to seven days in a high percentage of the treated animals. Roberts (17) has summarized the results of various investigators on corpus luteum enucleation by stating that estrus results in 50-80% of the cases treated within two to seven days, and that 50-55% of these cows conceive if bred. This method of altering the cycle holds little promise for routine use in estrous cycle regulation, since the incidence of adhesions in and around the oviducts appears to be increased as a result of the corpus luteum removal, an effect which may cause animals to be less fertile in subsequent years. Infrequently, a cow will die as a result of excessive hemorrhage after corpus luteum removal.

Many investigators have shown that daily injections of relatively large (25-100 mg.) doses of progesterone inhibit estrus and ovulation until four to seven days after cessation of the treatment (6). There is also general agreement that, although progesterone injections provide an effective method for cycle synchronization, the fertility of cattle bred at the first estrus after treatment is lowered (6, 23). The infertility appears to be temporary, since normal conception rates have been reported in cows bred at the second post-treatment estrus (6, 23).

Surprisingly, few attempts have been made to synchronize cycles in cattle with combined progesterone and gonadotrophin treatments. Nellor and Cole (15) reported a low conception rate (17%) in beef heifers given single injections of 560 mg. of crystalline progesterone, followed 15 days later by a single, subcutaneous injection of 2,140 I.U. equine gonadotrophin. Ninety per cent of these heifers came in estrus 16-19 days after the progesterone injection, or one to four days after the gonadotrophin injection. Donker *et al.* (4) cited evidence that progesterone pretreatment before gonadotrophin injections decreased the percentage of cows which ovulated after the gonadotrophin injections and increased the percentage which showed estrus.

Newer studies on cycle synchronization. Ray *et al.* (16) have recently reported poor synchronization and lowered fertility in beef heifers injected with 560 mg. of crystalline progesterone in a starch suspension, followed by 750-2,250 I.U. of PMS 14 or 15 days later. Estrus occurred 2-38 days after the PMS injection and 12% of the heifers conceived from insemination at this estrus. These workers also studied the effects of injections of Repositol[1] progesterone (0.76 mg. per pound body weight) at various stages of the cycle on pituitary FSH and LH potencies and on development of the endometrial glands. Pituitary FSH and LH potencies and endometrial gland cell heights were lowered in the treated animals. The average size of the corpora lutea was reduced in the treated animals. It was also suggested that the lowered fertility in progesterone-treated heifers may be due, in part, to subfunctional endometria.

Ulberg and Lindley (24) have recently reported on the effects of injecting various levels of estradiol benzoate 96 hr. after the last injection of a 14-day period of daily progesterone injections in beef cattle. After progesterone injections alone, 80.7% of 223 animals came in estrus within a ten-day period. Only 17% of these animals conceived when bred at the induced estrus. The percentage of cows in estrus during this ten-day period was increased (97% of 114 cows) in the cows which received estradiol benzoate (0.5-10.0 mg.) in

[1] Pitman-Moore Co., Indianapolis, Indiana.

addition to progesterone. Relatively few animals in this group came in estrus later than the fourth day after the last progesterone injection, although some of those receiving the higher dosages of estradiol came in estrus twice at about a 24-hr. interval. Injections of estradiol subsequent to the 14-day progesterone treatment did not cause a further reduction in fertility.

Hansel *et al.* (9, 11) attempted to produce synchronous estrus in Hereford heifers by utilizing several different treatments, each designed to bring the heifers in that particular treatment group in estrus during a predetermined target period. All animals were observed daily for estrus, beginning 30 days before the proposed target period. Those coming in estrus 23-30 days before the target period were recycled by oxytocin injections, as previously described (2). Those coming in estrus 19-23 days before the target period were allowed to cycle without treatment. Those animals coming in estrus later than 19 days before the target period were given seven daily subcutaneous injections of 130 U.S.P. units of oxytocin and daily subcutaneous injections of 50 mg. of progesterone in corn oil, concurrent with and subsequent to the oxytocin injections. The progesterone injections were discontinued four days prior to the target period. The three groups of heifers, all of which should, theoretically, have come in heat during the three-day target period thus consisted of: (1) heifers bred after a normal cycle following recycling by oxytocin injections, (2) untreated heifers bred after a normal cycle, and (3) heifers bred at an artificially induced estrus after combined oxytocin and progesterone treatment.

Results of this experiment are shown in Table 1, from which it may be seen that 26 of the 27 heifers were in estrus during an eight-day period consisting of the three-day target period, the two days prior to it, and the three days subsequent to it. The dispersion resulted primarily from the fact that 11 of the 13 oxytocin-plus-progesterone-treated heifers came in estrus during the three days after the target period.

Thirteen (50%) of the heifers coming in estrus conceived as a result of artificial insemination at this time. These included four of 13 oxytocin-progesterone-treated heifers, seven of nine untreated heifers, and two of four heifers bred at an estrus following a normal cycle initiated after recycling by oxytocin injections.

Hansel and Malven (10, 11) reported the first successful synchronization of estrous cycles in cattle as a result of feeding an orally effective progestational compound in 1960. In this experiment, 32 Hereford cows with nursing calves were bred artificially after feeding 6-methyl-17-acetoxy-progesterone,[2] a progestational compound which, in preliminary feeding experiments with Holstein heifers, had effectively inhibited estrus and ovulation until three or four days after its withdrawal. The compound was mixed in soybean oil meal and fed once daily. Approximately 4 lb. of this soybean oil meal was fed to each animal daily. The cows, which had an average weight of 1,000 lb., were group-fed and received approximately 968 mg. of the hormone per head per day for the first ten days and 500 mg. per head per day for the second ten-day period.

All animals were inseminated artificially three to five days after withdrawal of the progestational compound, whether or not they came in estrus. In addition, half of the cows, selected at random, were injected subcutaneously with 0.5 mg. of estradiol at the time of insemination.

Results of this experiment are shown in Table 2, from which it may be seen that none of the treated animals came in estrus until three or four days after withdrawal of the hormone, and 16 of the 32 cows showed estrus during this period. An additional 13 cows had ovulated by the fifth day without coming in estrus. Only 25% of the cows conceived to the treatment service, but it should be pointed out that eight of the 32 cows were bred after they had ovulated. The estradiol injections did not have any effect on fertility; equal numbers of estrogen-injected and non-injected cows conceived.

In a similar trial, conducted at the same time, oral administration of 187 mg. of 17-

[2] Provera, kindly provided by Dr. R. E. Gessert, The Upjohn Co., Kalamazoo, Michigan.

TABLE 1

Frequency of occurrence of estrus in Hereford heifers during, before, and after a target period

Treatment group	No. of heifers	Days [a]							
		−2	−1	1	2	3	4	5	6
No treatment	9	2	....	3	....	2	1	1	....
Recycled by oxytocin	4	....	2	1	....	....	....	1	....
Oxytocin plus progesterone	13	....	....	1	....	1	1	5	5
Total		2	2	5	....	3	2	7	5

[a] Days 1, 2, and 3 constitute the target period.

TABLE 2

Effects on reproductive performance of feeding 6-methyl-17-acetoxy-progesterone to Hereford cows for a 20-day period

Hormone fed	No. of animals	No. in estrus during treatment	No. in estrus 3 or 4 days after treatment	No. ovulating after treatment	No. conceiving to treatment service	Per cent conceiving to treatment service
6-Methyl-17-acetoxy-progesterone	32	0	16	29	8	25

ethynyl-5(10)-estrenolone (containing 1.5% 3-methyl ether of ethynyl estradiol)[3] was found to be ineffective in preventing estrus. Eleven of 18 cows came in estrus prior to the 20th day of the feeding period.

These encouraging results with 6-methyl-17-acetoxyprogesterone aroused considerable interest in the possibility of feeding orally effective progestational agents to regulate the estrous cycles in cattle, and subsequent experiments have shown that better synchronization and improved fertility can be obtained.

It was recently noted [Staples and Hansel (21)] that oxytocin-treated Holstein heifers which ovulated precociously without coming into estrus had little or no measurable progesterone in their corpora lutea. This indicated that a low level of progesterone may normally facilitate the manifestations of estrus in cattle, as had previously been suggested by Melampy *et al.* (13). These results suggested that the high proportion of cows which ovulated without coming in estrus after oral progesterone treatment in the previous experiment might have been the result of too low a level of progesterone after withdrawal of the hormone, rather than too high a level, as had previously been suspected.

As a result of this reasoning, 6-methyl-17-acetoxy-progesterone was fed to 20 Holstein heifers in the next experiment at the rate of 0.5 mg. per pound of body weight for 20 days, after which it was fed at a low level (0.05 mg. per pound of body weight) for an additional six days. These heifers were individually fed, and the required amount of hormone for each day was mixed in soybean oil meal at the rate of 30 mg. per gram and placed on top of each animal's daily grain ration.

Results of the experiment are shown in Table 3, from which it may be seen that 18 of the 20 heifers were in estrus on the third and fourth days after the level of progesterone in the feed was reduced. Ten of the 18 heifers conceived when bred artificially at the induced estrus.

The percentage of animals coming in estrus and the fertility were both improved over the previous trial, but a comparison is scarcely justified, since the first trial involved group feeding of Hereford cows nursing calves and the second involved individually fed Holstein heifers.

The optimum level at which 6-methyl-17-acetoxy-progesterone should be fed has not yet been well established. Early experiments of Hansel and Malven (10) and Nellor *et al.* (14) suggested that the level necessary to inhibit estrus and ovulation lies between 0.25 and 0.50 mg. per pound of body weight per day, and that ovulation without estrus is likely to occur on levels as low as 0.20 mg. per pound of body weight.

Zimbleman, however, (26) has recently reported successful synchronization of cycles in Holstein heifers fed 180-400 mg. of the hormone in two feedings daily for 20 days. These

[3] Enovid, kindly supplied by Dr. G. E. Short, The G. D. Searle Co., Chicago, Illinois.

TABLE 3

Effects on reproductive performance of feeding Holstein heifers 0.5 mg. per pound body weight of 6-methyl-17-acetoxy-progesterone for 20 days followed by 0.05 mg. per pound body weight for 6 days

Hormone fed	No. of animals	No. in estrus during treatment	No. in estrus 3 or 4 days after dose lowered	No. ovulating after treatment	No. conceiving to treatment service	Per cent conceiving to treatment service
6-Methyl-17-acetoxy-progesterone	20	0	18	18	10[a]	56

[a] Two additional heifers were diagnosed as pregnant on the 44th day, but returned to estrus and were found not pregnant on the 55th and 59th days after breeding.

levels range from 0.20 to 0.45 mg. per pound for heifers weighing 900 lb.

Thirty-seven of 40 heifers that came in estrus did so between noon of the second day and noon of the fourth day after hormone feeding was stopped. Six additional heifers ovulated without showing estrus.

Conception rates obtained in this experiment are shown in Table 4, from which it may be seen that acceptable fertility was obtained with levels as low as 180 mg. per day, or approximately 0.20 mg. per pound of body weight.

On the other hand, feeding higher levels of the hormone does not seem to cause decreased fertility. Nelms (personal communication) fed a much higher level of 6-methyl-17-acetoxy-progesterone (0.8 mg. per pound of body weight per day) in two feedings per day for 14 days to ten Hereford cows. Nine of the ten came in estrus three to five days after the hormone was withdrawn and the remaining cow came in estrus on the seventh day. Seven of the ten cows conceived when bred at this estrus.

The stage of the cycle at which feeding of the oral progesterone is begun does not appear to influence either the length of time elapsing from the end of the feeding period to estrus or the fertility of the heifers bred. Collins *et al.* (3) fed 6-methyl-17-acetoxy-progesterone at the rate of 0.5 mg. per pound of body weight daily for 20 days, beginning on Days 3, 7, 11, 15, and 19 of the cycle. Thirty-five of 36 heifers were in estrus two to five days after cessation of the hormone feeding. The interval to estrus was not affected by stage of the cycle at which the treatment was begun. Twenty-two heifers were artificially inseminated and 13 were diagnosed pregnant 34 days later. Fertility was not affected by the stage of the cycle at which treatment was started.

The question of the optimal length of time to feed the hormone is also unsettled. Theoretically, a minimum of 20 days' feeding would be necessary to inhibit estrus in all animals until the corpus luteum regressed. Actually, it may be possible to produce effective synchronization of cycles by feeding an oral progesterone for shorter periods of time.

Obviously, the feeding period could be reduced to ten days if the development of the corpus luteum could be inhibited in all animals in a given group that were within the first five days of their cycle at the beginning of the feeding period. This can be accomplished either by oxytocin injections or by uterine dilatation, as previously discussed.

To test this possibility, 15 Holstein heifers were fed 0.4 mg. per pound of body weight of 6-methyl-17-ocetoxy-progesterone once daily in their grain ration, as previously described, for ten days. They were then fed the hormone at a reduced level (0.05 mg. per pound of body weight) for an additional five days. Two of the 15 heifers had been in estrus within five days before the beginning of the feeding period, and self-retaining catheters were placed in the uteri of these heifers, as previously described (12), on the first day of the trial. All 15 heifers were given single subcutaneous injections of 100 U.S.P. units of oxytocin on the first day of the trial. Results of this experiment are shown in Table 5.

Thirteen of the 15 heifers were in estrus on the third day after the dose of the oral progesterone was lowered. One heifer came in estrus on the fourth day, and the remaining one on the fifth day. All heifers were bred artificially in the usual way, and all ovulated after the artificially controlled estrus. Five of the 15 heifers returned to estrus 18 to 24 days after insemination, and nine of the remaining ten were diagnosed pregnant on the 44th day. The remaining heifer returned to estrus on the 45th day.

The two heifers with catheters in their uteri expelled them on the first and second days, respectively, after the hormone dose was lowered, and both were in estrus on the third day. One of the two heifers conceived.

Uterine dilatation plus progesterone feed-

TABLE 4

Conception rate to first service at the synchronized estrus in dairy heifers [a]

Average daily dose of 6-methyl-17-acetoxy-progesterone	How fed	No. of heifers		Per cent conception
		Inseminated	Conceived	
180	Group	7	4	57
210	Group	7	5	71
400	Group	8	6	75
360 to 480	Ind.[b]	8	2	25
	Total	30	17	Average 57

[a] Data kindly supplied by R. G. Zimbelman (26), The Upjohn Co.
[b] Penned individually twice daily for feeding.

TABLE 5

Effect on reproductive performance of Holstein heifers of a combined treatment involving oral progesterone feeding and uterine dilatation

Hormone fed [a]	No. of animals	No. in estrus during treatment	No. in estrus 3-5 days after dose lowered	No. ovulating after treatment	No. conceiving to treatment service	Per cent conceiving to treatment service
0.40 mg/lb body weight 6-methyl-17-acetoxy-progesterone for 10 days followed by 0.05 mg. per pound body weight for 5 days	15	0	15	15	9	60

[a] In addition, self-retaining uterine catheters were placed in the uteri of all heifers in the first five days of their cycle at the beginning of the hormone feeding period. All heifers received single injections of 100 U.S.P. units of oxytocin at the beginning of the hormone feeding period.

ing appears to provide an effective method for synchronizing the cycles of Holstein heifers in a short time. The method as used to date requires that the heifers be checked for estrus for five days before the beginning of the feeding period to determine those in which the uterus must be dilated. In mature cows it may be possible to modify the method to the extent of placing catheters in the uteri of all cows, regardless of the stage of cycle. Oxytocin injections, coupled with feeding of an oral progesterone, should be an equally effective method for synchronizing cycles in a short time, but a simplified method for administering the oxytocin in a single injection would be necessary to make the method practicable.

Although considerable additional work must be done to determine the exact method which will give optimal results, it seems safe to say that practical methods are now available for artificially regulating the estrous cycle so as to produce synchronous estrus in groups of heifers.

Although several of the recent trials indicate that nearly normal conception rates can be obtained after cycle synchronization, none of the experiments conducted to date has been designed to adequately test the fertility of the treated animals. Understandably, most of the emphasis until now has been placed on developing workable systems for cycle synchronization. Properly controlled experiments involving larger numbers of animals are now needed to test the fertility of cattle bred at synchronized cycles.

The extent to which these methods are applied will depend primarily upon the cost of the hormones which must be used, and the minimal dose levels and feeding periods finally established.

The problem of anestrus. No discussion of estrous cycle and ovulation control in cattle would be complete without some consideration being given to the causes of failure of cows and heifers to come in estrus. This is one of the most difficult and costly problems confronting dairymen and veterinarians today. Although there may be many causes of anestrus, our knowledge of the causes can be conveniently divided into three general categories.

The first of these is a failure of heifers to reach maturity and initiate estrous cycles at the expected age, because of underfeeding. The data of Sorenson *et al.* (20), among others, clearly illustrate this point. These workers found that the average ages at first estrus of heifers grown on levels of feed intake corresponding to 61, 93, and 129% of recommended TDN allowances were 72, 49, and 37 wk., respectively.

Factors causing lowered hemoglobin level in mature cows constitute a second cause of anestrus. Any factors which result in hemoglobin levels of approximately 8.0 mg. % or lower are likely to cause prolonged periods of anestrus (Hansel—unpublished observations). In New York State three causes of lowered hemoglobin levels and anestrus have been found: cobalt deficiencies, associated copper and cobalt deficiencies, and heavy infestations of lice. There are undoubtedly other nutritional deficiencies that can cause anestrus, but their occurrence under farm conditions has been more difficult to demonstrate.

The third kind of anestrus animal is a great deal more prevalent and probably less well understood than the others mentioned. This is the typical cow that does not come in estrus for prolonged periods after calving. Many of these animals have corpora lutea formed after calving, apparently as a result of ovulations not accompanied by estrus [Trimberger and Fincher (22)]. In the light of what has already been said, it is tempting to

speculate that the first ovulation in cows of this type is initiated by some form of uterine dilatation or irritation, and that estrus does not accompany the ovulation in a high percentage of cases because of the absence of significant amounts of progesterone of luteal origin at this time.

Once ovulation has occurred in cows of this type, the corpus luteum may persist (and apparently remain functional) for long periods of time. Estrus does not occur and the enlarged uterine horns become filled with products of inflammation and lose tone.

This situation may well be the result of a failure in the normal functioning of the same mechanism by which uterine dilatation and oxytocin injections inhibit development of the corpus luteum and provoke precocious estrus and ovulation.

Prolonged periods of anestrus of more than 150 days and maintenance of corpora lutea result from hysterectomy in the cow [Wiltbank and Casida (25)]. Furthermore, oxytocin injections fail to induce estrus in cattle from which all of the uterus except the cervix is removed [Armstrong and Hansel (2) and Melampy, personal communication].

Anderson (1) has recently studied the influence of removal of various parts of the uterus on the occurrence of estrus in Hereford heifers. When the entire uterus, including the cervix, was excised, corpora lutea were maintained and estrus was inhibited in four heifers for 270 days. The corpora lutea regressed and three of four heifers returned to estrus when both uterine horns were excised and the cervix and body of the uterus were left. Similarly, the corpora lutea regressed and estrus occurred in four of four heifers in which the anterior fourth of each horn was left intact and in four of four heifers in which the anterior half of each horn was left intact. Data obtained to date indicate that the presence of the cervix alone is not sufficient to cause luteal regression and the recurrence of estrus. However, the presence of any other part of the uterus does result in luteal regression and estrus.

These data are all consistent with the idea that in the anestrus cow with a persistent corpus luteum, afferent stimuli of uterine origin fail to arise in the normal way and act through the hypothalamus to shift the pattern of pituitary gonadatrophin secretions in such a way as to cause luteal regression, follicle growth, estrus, and ovulation.

However, another interpretation of the data, which has some experimental support, is possible. Duncan *et al.* (5) have revived the old discussions of the possibility of a uterine hormone by showing that filtrates of endometrial tissues taken from gilts at the 12th and 13th days of the estrous cycle increased the in vitro synthesis of progesterone by luteal tissue, whereas similar filtrates taken at the 16th-18th days of the cycle inhibited in vitro progesterone production. However, no one has yet devised an experiment in which extracts of uterine tissues have been shown to have a clear-cut luteolytic or leuteotrophic effect in an in vivo situation.

This question of whether the uterus exerts its effects on the corpus luteum and the estrous cycle by neurogenic or humoral means is further complicated by the lack of specific information concerning which gonadotrophin (if any) is responsible for maintenance of the corpus luteum in the bovine. It has not been possible to maintain the corpus luteum and prolong the cycle in cattle by daily injection of prolactin of ovine origin (19).

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ARTIFICIAL INSEMINATION OF CATTLE

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Artificial insemination offers one of the most effective means of improving reproductive efficiency. Although making improvements in reproductive efficiency in the cow population through artificial insemination in most respects is not much different than in natural mating, the potential with the male is enormous. Maximum reproductive efficiency is interpreted to mean—getting the maximum number of calves from the parent stock per unit of time.

To be sure, artificial insemination may be useful in insuring protection from disease for the cow, thus permitting a longer useful life and more frequent calving. However, earlier calving and subsequent shorter calving intervals through shorter gestation periods and optimal post-partum breeding intervals are usually as readily obtainable by natural mating as with artificial insemination (34). With the male, on the other hand, artificial insemination brings with it the possibilities of increasing the offspring of a desirable sire several hundred-fold.

Consider expressing reproductive efficiency by an equation proposed by the Cornell workers (14) some time ago:

$$\text{Calves per bull per year} = \frac{\text{No. live sperm produced}}{\text{No. live sperm per insemination}} \times \% \text{ semen used} \times \% \text{ cows calving}$$

From this, we can readily see the role played by artificial insemination in increasing reproductive efficiency is primarily through increasing the utilization of sperm produced by the bull. Maximum sperm production by bulls is still not fully realized, but great strides have been made in recent years in describing and defining many of the factors affecting sperm production. Likewise, great strides have been made following the early Cornell studies on limits of extending semen (25, 27, 28, 31). The next item in the equation, per cent of semen used, was for many years, and under certain conditions still is, a great deterrent to the full realization of a bull's potential, because of the loss of semen through aging. The last item of the equation has been left to the other speakers of this symposium, except as that factor is dependent upon the other factors of the equation for optimal conception rates.

In 1957, Foote and Bratton (14) integrated the above-mentioned factors and presented a table, partly reproduced in Table 1, to show

TABLE 1

Interrelationship of factors affecting the possible number of progeny produced [a]

Motile sperm per bull per year	No. of motile sperm used per cow	Per cent of sperm used	Per cent conceiving (one insem.)	No. of progeny
(million)				
250,000	50	25	50	625
250,000	50	25	70	875
250,000	10	25	50	3,125
250,000	10	25	70	4,375
250,000	10	50	50	6,250
250,000	10	50	70	8,750
750,000	10	50	50	18,750
750,000	10	50	70	26,250
750,000	5	50	65	48,750

[a] Modified from Foote and Bratton (14).

the potential offspring of a bull under conditions resulting in changes in efficiency in the several items of the equation. This table does not show the even lower number of offspring—perhaps 25 to 50—that would result from natural mating. Sperm production is considered from 250 billion motile sperm per year, a reasonable estimate for a mature bull producing one ejaculate per week, to 750 billion, a conservative estimate in view of later evidence. Sperm used per insemination covers the range from early use, 50 million motile sperm per insemination or about a 1:20 extension rate, to five million motile sperm per insemination, a level established in the early experiments that appears to be near the minimum even today. The percentage of semen utilized is considered from 25%, a realistic picture in some operations even today, to 50%, a level still far from maximal. Finally, the conception rates shown are realistic of the changes that occur in many A.I. organizations as they go through the trials of early growth. Although units frequently start with conception rates as low or lower than 50% (60- to 90-day nonreturns), conception rates of 70% and above have been obtained consistently by some units performing thousands of inseminations. Still, in the United States in 1960, the average A.I. sire was used to inseminate only about 3,000 cows.

Additional evidence in several areas suggests that changes are possible that will push the potential offspring of a bull many times beyond that now being obtained and even beyond the projections shown in Table 1. No attempt has been made here to cite all the references pertaining to the items discussed. However, the reviews and papers mentioned do refer to many other pertinent references.

INCREASING SPERM PRODUCTION

One of the obvious limitations to the number of calves that can be sired by a particular bull is the number of sperm produced by the bull. Extending the useful life of a bull is one way of increasing his potential of siring offspring. In recent years, it has been shown also that sperm production can be increased by more intense preparation and stimulation before collection and by more frequent collections of semen.

Extending the useful life of bulls. In 1954, Becker *et al.* (6) showed that the average tenure of a bull in artificial insemination service was only 2.72 yr. Yet they showed that the anticipated further useful life of a 5-yr.-old bull in natural service was over 5 yr. This suggests that bulls in A.I. service were not lasting as long as they should. As shown in Table 2, few bulls went out of A.I. service

TABLE 2

Reasons why bulls go out of A.I. service [a]

Reason	Lost
	(%)
Low fertility, sterility, poor semen, or refusal to work	61.1
Poor physical condition (including bloat)	11.0
Infectious causes	8.3
Accidents and injuries	7.1
Old age	4.0
Other unidentified or unstated	8.5
Total	100.0

[a] Compiled from Becker *et al.* (6).

because of old age, but many went out for reasons such as accidents, injuries, and infections, which careful management should help to prevent. The greatest losses came from low fertility, sterility, poor semen production, and refusal to work. Some of the research reviewed below has probably helped to avoid some of the losses in this category, but far too little emphasis in research today is directed at avoiding or correcting low fertility and sterility.

Becker (5) recently pointed out that, since 1954, bulls have remained in A.I. service for an average of over 4 yr. This apparently means that greater care has been used in selecting and maintaining bulls in A.I. usage. Perhaps more young bulls have been taken into service and fewer old bulls that will meet the rigors and standards of A.I. have been pressed into A.I. usage.

There are some suggestions that the useful life of bulls may be extended by getting bulls started at an earlier age. This is indicated by work from a number of quarters that shows rapid early growth brings on early semen production (see reviews in References 23 and 34). However, such studies need to be continued throughout the lifetime of bulls, to be certain that rapid growth and early maturity do not shorten the useful life.

Rapid early growth and semen production. During the growing period there is a high correlation between body size and testes size [$r = .90$ for 65 bulls (37)] and between testes size and semen production [$r = .80$ for 11 bulls (37), and $r = .92$ for nine bulls (46)]. This high degree of relationship, however, disappears in mature bulls (46), probably as a result of the ravages of disease and other factors that tend to inhibit testes function. One of the first noticeable effects of retarded growth on reproductive function is shown by the delay in puberty. This is shown in Figure 1, where the effect of varying levels of total digestible nutrient intake during early growth in trials at three stations have been brought together and plotted against age at first semen production (8, 12, 42).

Retarding growth apparently results not only in delayed puberty and low sperm production early in life, but from limited investigations it appears that irreparable damage may be done if the retardation is continued through the normal growing period. Illinois workers found no recovery of semen-producing ability of Holstein bulls in a full year with normal feeding following a 4-yr. period when the bulls were kept on a ration containing only 60% of the recommended TDN requirements (41). Thus, it appears that further investigations are needed to determine whether rapid early growth does increase a bull's lifetime sperm-producing potential.

Adequate preparation for collection. A number of studies in recent years, following earlier suggestive evidence (reviewed in References 17, 34, and 37), have shown that semen volume and sperm numbers can be increased considerably by adequate preparation for collection.

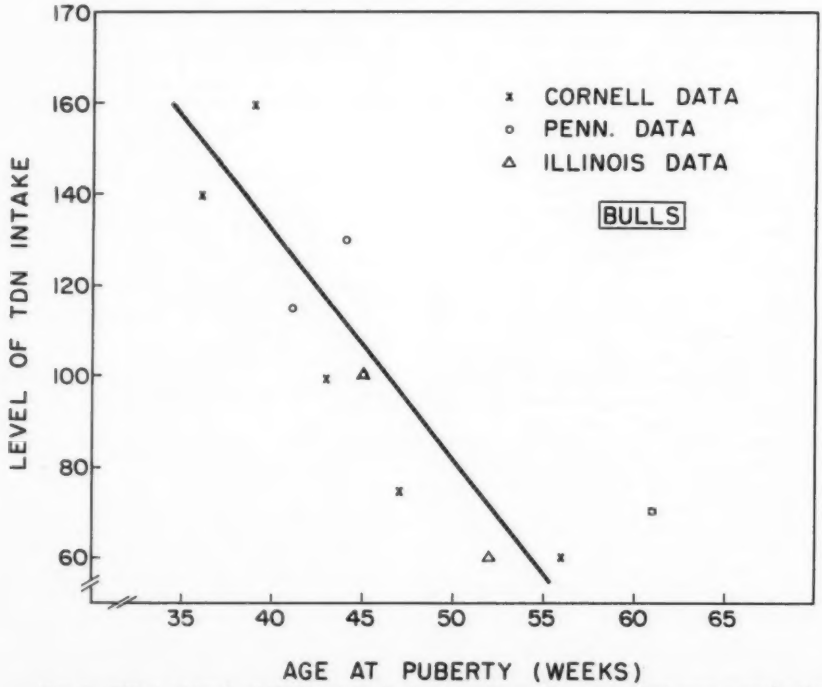

FIG. 1. Effect of level of TDN intake during growth on the age at first semen production of Holstein bulls (regression equation $Y = 65.22 - 0.1878X$).

Both restraint near the teaser cow and false mounting prior to collection bring on sexual excitement resulting in increased yields of sperm. A number of the studies in this area have been compiled in Table 3. Increases in sperm output in these studies have ranged from 14 to as much as 250% over the number of sperm produced in ejaculates when the same bulls were allowed to work at will.

Studies at Pennsylvania have done much to show that changing teasers and changing the position of the teaser greatly aid in stimulating a bull to work (17). Their studies have shown that bulls apparently depleted of sperm in exhaustion trials are frequently stimulated to produce sizable quantities of semen when a new stimulus (teaser) animal is supplied. Maintaining sex drive seems to be more of a limiting factor to the production of large numbers of sperm than a shortage of sperm production by the testes.

More frequent collection. Increasing the frequency of collection was one of the first approaches to obtaining larger numbers of sperm from bulls. For many years it was common practice to limit collections to one or two ejaculates every week or two. It was believed that more frequent collection would result in lowered fertility or sterility. This belief no doubt stems from pasture breeding, where ten to 20 services per cow in heat are not unusual for young, vigorous bulls (45). Thus, with a fairly large herd the ejaculation frequency would soon deplete the sperm reserves and sex drive would likely be diminished.

Controlled investigations from a number of places have changed the old view with regard to collection-frequency effects on sperm output. Collection frequencies of two and three times per week greatly increase the sperm output of old and young bulls alike. Even with exhaustive collections (see Table 4), the depleted sperm supply is largely replenished 1 wk. after depletion. Rather frequent collections are possible without lowering fertility. As seen in Table 5, taken from Hale and Almquist (17), collections daily or six days a week for 26 to as long as 76 wk., have yielded large numbers of sperm (enough to breed more than 500 cows a day), with fertility levels in the ideal range of 70 to 81% 60- to 90-day nonreturns. Other work at Pennsylvania, in which six (twice on Monday, Wednesday, and Friday), seven (daily), 14 (twice daily), or 70 (ten daily) ejaculates per week were collected, showed that six or seven ejaculates per week withdrew nearly as many sperm as the testes produced. More frequent collections increased sperm output slightly. Thus, sperm production should be considered on the basis of the bull's potential output, rather than on a prescribed number of collections per unit of time. On such a basis, sperm collections can be made with the artificial vagina or with electroejaculator equipment, even though some may consider the latter an undesirable method, without stopping far short of a bull's potential or exceeding it.

NUMBER OF SPERM TO INSEMINATE

The question of the number of sperm required per insemination was first considered

TABLE 3

Effect of sexual preparation on total sperm output

Investigator(s)	Type of preparation	No. of bulls	No. ejaculates per week	Per cent increase: First ejaculate	Per cent increase: Second ejaculate
Collins *et al.* (9)	None vs. 2 to 3′R[a] + 1 FM	22	1	36	....
Branton *et al.* (7)	None vs. 1 FM[b]	6	1	42	....
	None vs. 2 FM	6	1	41	....
Branton *et al.* (7)	None vs. 1 FM	9	2	50	....
	None vs. 2 FM	9	2	67	....
Baker (4)	None vs. 1 FM	9	1–3	22	....
	None vs. 3′R	9	1–3	14	....
Crombach (10)	None vs. 1 FM	6	4	129	....
	None vs. 10′R	2	4	147	....
	None vs. 5′R + 1 FM	4	4	251	....
	None vs. 5′R + 1 FM	10	4	112	109
Almquist *et al.* (2)	None vs. 1 FM	6	2	72	29
	1 FM vs. 2′R + 3 FM	6	2	64	27
	None vs. 2′R + 3 FM	3	6	....	45
	1 FM vs. 2′R + 3 FM	3	6	44	....

[a] R = restraint.
[b] FM = false mount.

TABLE 4

Recovery of semen characteristics 1 wk. after depletion [a]

Reference	No. of bulls	No. of trials	Av. No. ejac. in depletion	Av. No. ejac. in recovery	Per cent recovery of: Semen volume	Sperm motility	Sperm concentration	No. of sperm per ejac.
Hale and Almquist	26	46	24	2	84	99	103	86
Hale and Almquist	13	17	22	5	84	100	112	94
Frederick	6	6	24	21	77	92	105	85
Boyd and VanDemark	6	6	10	10	74	96	145	100

[a] From Hale and Almquist (17).

TABLE 5

Fertility of bulls with high sperm output over extended periods [a]

Bull	Weeks	Frequency of ejaculation	Daily sperm output	60- to 90-day nonreturns	First services
Hafs *et al.* (Cornell)			*(billions)*	*(%)*	
D	32	Daily	2.7	81	531
B	32	Daily	5.6	71	1,795
S	32	Daily	5.7	72	3,038
H	32	Daily	5.9	70	982
C	32	Daily	4.3	72	762
Almquist *et al.* (Pennsylvania)					
G[1]	76	6/Week	4.5	70	544
H[11]	32	6/Week	4.8	74	286
H[44]	50	6/Week	6.0	78	881
[b]	26	6/Week	7.4 [b]	79	1,709

[a] From Hale and Almquist (17).
[b] Prepared with three false mounts before each ejaculate.

by Salisbury and associates at Cornell in the early and mid-forties, with a series of experiments in which they attempted to establish maximum extension rates for bull semen (25, 27, 28, 31). As a result of those studies, Salisbury and Bratton (28) wrote that "the minimum number of spermatozoa consistent with optimum fertility rests at 5 to 10 millions from bulls of known fertility."

Subsequent investigations by a number of workers have not altered these original estimates of minimal sperm numbers for fertility in artificially inseminating the cow. Figure 2 shows the results of several of these investigations (34). From these it is apparent that a sharp drop in fertility occurs if the number of motile sperm per insemination is reduced much below five million, but increasing the number of live sperm above five million does not result in corresponding increases in fertility.

PERCENTAGE OF SEMEN UTILIZED

To take advantage of the large sperm-producing capacity of a bull, and to breed many cows with a single ejaculate by extending the semen so that each cow gets only a limited number of sperm, good methods of semen preservation must be available. This is another area in which great improvements in efficiency have been made in the last 20 yr. The main problem in getting a high percentage of semen utilization is in maintaining viability and fertility over an extended period of time, to permit distribution of the semen and hold it in readiness for the presentation of estrous cows for service.

Two main approaches taken to preserve semen have been that of reducing temperature to slow metabolism and motility and that involving reversible chemical inhibition, or combinations of the two. A third method, lyophilization or freeze-drying, also appears possible and suggests some promise. Supplying diluents that help to maintain optimal conditions by furnishing nutrients and removing waste products also greatly prolongs the storage life of spermatozoa. Salisbury (26) reviewed the status of bovine semen diluents in Animal Breeding Abstracts in 1957. A later and more detailed consideration of the merits of various diluents can be found in the recent book of Salisbury and VanDemark (34).

Storage at refrigerator (above-freezing) temperatures. The wide-spread use of cooling to refrigerator temperatures to prolong sperm life in this country dates back to about 1940. In fact, the protection from cold shock af-

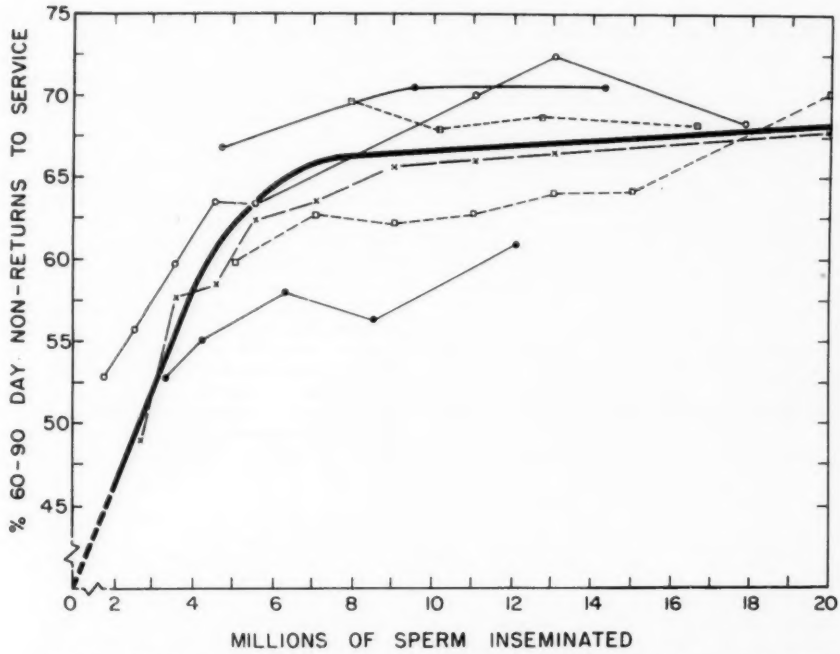

FIG. 2. Effect of number of sperm inseminated on fertility of dairy cows. (From Physiology of Reproduction and Artificial Insemination of Cattle, by G. W. Salisbury and N. L. VanDemark. W. H. Freeman and Company, San Francisco. 1961.)

forded sperm by the yolk diluents [Phillips' yolk-phosphate (22) and the Salisbury *et al.* yolk-citrate (32)] permitted cooling and greatly lengthened the useful life of semen. Even with a storage life of three or four days on a routine basis, some A.I. operations poured down the drain over 80% of the semen collected and processed. Addition of antibacterial agents improved storage life by eliminating much of the competition of bacteria and improved fertility, too (3, 19). A good example of the effects of antibacterial agents is seen in the results presented by the Cornell workers, comparing fertility results before and after addition of antibacterial agents (13). During a 6-mo. period before antibiotics, fertility averaged 64%; in the 6 mo. after antibiotics were added, fertility rose to 73% (60- to 90-day nonreturns).

The presence of antibiotics and sulfanilamide in extended semen results not only in slightly better conception rates, but fewer cows return to estrus at irregular intervals after unsuccessful artificial insemination (21). Additions of penicillin to semen have been shown to increase the fertility of some low-fertility bulls (1). Little is known of the actual effect of semen processing and handling procedures on fertility. As semen ages in storage at refrigerator temperatures, and other temperatures, too, there is a decline in the fertility (34, 35). It has been shown that DNA (the chemical substance associated with the genetic potentials of the sperm) is lost during storage at refrigerator temperatures (30). This loss may be responsible for the gradual increase in apparent embryonic death that occurs following the insemination of cows with semen stored over longer and longer periods (29).

It is tempting to postulate that storage conditions may be responsible for such phenomena as decreased fertility and increased embryonic death, but the proof is not yet at hand. However, such items as the oxygen damage that occurs in sperm in partially filled tubes (43), and when semen is mixed excessively and aerated (a damage that can be prevented by the use of catalase), indicate a possible oxidative damage which could involve the DNA.

Many improvements in semen diluents have been made which enhance the effectiveness of reduced temperatures in slowing metabolism and prolonging sperm survival (34).

Storage at room temperatures utilizing chemical inhibition. One new approach to semen preservation, made in the last 10 yr., was the attempt to simulate the conditions of the epididymis and keep sperm in a constant-flow

dialysis system (40). Although unextended semen lived for over a week under such conditions, compared to a day or less in the test tube at room temperature, the real beneficial discovery from those studies was the effect of carbon dioxide on sperm. Carbon dioxide in concentrations above those normally found in extended semen reduces metabolic activity of spermatozoa and inhibits motility (33). Out of such studies was developed the carbonated Illini variable temperature (IVT) diluent (44). Under conditions in which the ambient temperature does not rise for too long above 80° F., this type of diluent, saturated with carbon dioxide, has maintained quite good fertility for two or three days. However, under conditions where the temperature remains long above 80° F., and where care is not taken to keep high levels of CO_2 in the diluent, disappointing results have been obtained.

Combining chemical inhibition and reduced temperatures. Advantage has been taken of reversible chemical inhibition while eliminating the difficulties encountered at ambient temperatures by refrigerating semen extended with the carbonated diluents. A number of such diluents, all of rather similar composition, are shown in Table 6. All except the New York CUE are gassed with CO_2. The CUE is self-carbonating, generating CO_2 from the mixture of sodium bicarbonate and citric acid.

Fertility levels published from the use of some of these diluents are shown in Table 7. All attempts to use this method of semen preservation have not been as successful as those shown, but the poorer results are known to have been due, in some cases at least, to improper procedure and care in keeping the CO_2 in the storage ampule or tube.

Preservation by freezing. Freezing exceeds all other developments to date for the preservation of bovine semen. The British have

TABLE 6

Some carbonated diluents for bovine semen[a]

Ingredients	Orig. IVT	Mod. IVT	Ark. No. 2	Tenn. YCCG	Germany EIBL	N. Y. CUE
Sodium bicarbonate	0.21	0.42	0.21	0.21	0.18	0.21
Sodium citrate	2.00	1.00	1.00	1.60	1.67	1.45
Potassium chloride	0.04	0.04	.....	0.04	0.03	0.04
Glucose	0.30	1.20	0.30	1.00	0.25	0.30
Sulfanilamide	0.30	0.30	0.30	0.30	0.25	0.30
Egg yolk *(%)*	10	15	10	20	30	20
Penicillin *(I.U/ml)*	1,000	1,000	1,000	500	500	1,000
Streptomycin *(μg/ml)*	1,000	1,000	1,000	500	500	1,000
Catalase	.....	0.01	.....	.....	.....	.....
Glycine	.....	.....	1.00	.....	.....	0.94
Glutathione (reduced)	.....	.....	0.154	.....	.....	.....
Citric acid	.....	.....	.....	.....	.....	0.087
Gassed with	CO_2	CO_2	CO_2	CO_2	CO_2	.....
Cited from References	(44)	(39)	(18)	(36)	(11)	(15, 16)

[a] Grams per 100 ml. unless otherwise indicated.

TABLE 7

Per cent 60- to 90-day nonreturns from inseminations with carbonated diluents compared with control diluents

Age of semen	Investigation and diluents used					
	Arkansas		Tennessee		New York	
	CO_2 No. 2	YC	YCCG	MYGLy	CUE	YC
(days)						
0-1	78	74	....	....	....	....
1-2	76	68	75	78	78	75
2-3	72	62	78	72	75	72
3-4	66	60	75	70	73	67
4-5	63	....	71	69	....	....
5-6	63	....	....	....	....	....
6-7	67	....	....	....	....	....
Over-all	70.9	66.6	74.4	72.6	76.6	73.4
No. cows bred	8,657	1,481	2,015	1,974	6,280	6,069
Cited from References	(18)		(36)		(15)	

gotten pregnancies from semen stored over 8 yr. (24) and we must be near or exceeding that in the United States by now.

Although the period of storage can be long, thus making possible high percentage utilization, use of the freezing technique is not without wastage (see Review References 34, 38). In the first place, semen of some bulls just will not come through the freezing process without a high loss of motile sperm. Secondly, even with the semen that does freeze fairly well, there is a sizable loss in motile sperm resulting from freezing. And, lastly, there is a loss of motile sperm during storage in the frozen state. To date, no special treatment is known to improve the freezability of semen from hard-to-freeze bulls or to eliminate the 20 to 30% kill commonly encountered in freezing. However, holding the storage temperatures much below the danger point by means of liquid air or liquid nitrogen has helped to reduce loss of motile sperm during storage in the frozen state.

From the practices being followed by those using frozen semen, it appears that a liquid semen program that utilized 50% of the semen would be as efficient at a lower cost than an all-frozen semen program. However, a number of other items in addition to utilization of bull power must be considered, as well, in deciding between a liquid and frozen semen program.

Freeze-drying for semen preservation. The dream of researchers, and practical men as well, that semen might be preserved indefinitely in a dry, unrefrigerated state after lyophilization or freeze-drying, has recently appeared hopeful. Some of the early reports of success with this technique with laboratory and farm animals in Russia were recently reviewed (38). The report of Meryman and Kafig (20), of the successful impregnation of a cow in Maryland with freeze-dried sperm, appears to be the first such report in cattle. Unfortunately, with this procedure, as with many new and revolutionary techniques, the original investigators and others have not yet succeeded in repeating this feat. So, once again, perfection of the procedure may take months or even years before it becomes practical. However, now it would appear to be the ideal method of semen preservation and utilization.

THE CHALLENGE

Even without using freezing, or awaiting the perfection of freeze-drying as a semen preservation technique, the potential of most bulls would appear to be far greater than many artificial organizations are utilizing at the present. It appears, from the investigations cited above and hoping for nearer 100% utilization of the semen produced, that the following values inserted into the formula for reproductive efficiency (given earlier) may be reachable at least for some bulls:

$$\frac{5 \text{ billion live sperm/day} \times 300/\text{yr}}{5 \text{ million live sperm/insem}} \times 100\%$$

usage $\times$ 75% calving = 225,000 calves/yr.

Semen production and an operation at this level of efficiency is a real challenge to the cattle artificial insemination industry. An even greater challenge is that of selecting bulls of genetic worth that should be used as extensively as such an operating efficiency would permit.

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PRESENT STATUS OF THE REPEAT-BREEDER COW PROBLEM [1]

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It is necessary for purposes of mutual understanding to define the term repeat-breeder. Such a cow, as considered in this discussion, is normal in a gross sense and gives no clinical evidence of infectious genital disease. Palpation of the genital organs shows no gross evidence of anatomical abnormality or of existing inflammation, or persisting pathology. Also, neither hyper- nor hypo-ovarian activity is detectable. The animal has a history of reduced fertility, but the cause of it is undiagnosed. A practice often followed in selecting animals for experimental purposes has been to choose cows that have returned from a certain number of services, usually four or more. This reviewer sees no good reason, however, if semen quality and insemination circumstances are optimal, for the animal not qualifying as a repeat-breeder upon a single return from service. A repeat-breeder, then, other factors being optimal, is a cow which shows a reduced probability of conception.

FIRST-SERVICE FEMALE AND CLINICALLY NORMAL REPEAT-BREEDER COMPARED

We are at loss to know today what information to obtain about clinically normal individual animals that will give any material advantage over the average chance in predicting whether a cow is a potential repeat-breeder. Even the most highly selected normal first-service heifers as a class will show some repeat-breeding. A comparison of the performances of clinically normal repeat-breeders and clinically normal first-service heifers is in order. No study has been reported in which these different kinds of animals have been surveyed simultaneously; such a study, however, is being made at Pennsylvania. A comparison of animals from different studies is presented in Table 1.

[1] Paper No. 833 from the Division of Genetics.

Some abnormalities of the reproductive tract are found both in first-service animals and repeat-breeders. Although they represent the inability of the investigator to identify the conditions in the living animal, their proportion appears to be highest in the repeat-breeder heifers.

The next category in the table is complicated by the inclusion of animals from which no eggs were recovered in the laboratory. There is no known reason why the technical error in recovery should be greater with one class of animal than with another; therefore, it is presumed that observed differences in this category represent actual increases in fertilization failure in the repeat-breeder.

Increased amounts of embryonic loss also occur in the repeat-breeder. The ratio of the third category to the sum of the last two categories represents the proportion of embryos lost; this, obviously, is much greater for the repeat-breeder. One suspects, however, that the problem for the whole population of repeat-breeders is almost equally an increase in fertilization failure, with its various causes, and an increase in embryonic loss.

THEORETICAL CAUSES FOR REPEAT BREEDING

On theoretical grounds, the causes of repeat-breeding are traceable either to defects of the egg or embryo or to defects of the maternal environment. There are two different points of view of long standing on the defects of the maternal environment: (1) The sterility is due to the presence of a low-grade, nonspecific infection in the female genital tract and (2) the sterility is of a functional type and involves endocrine controls and various physiological functions of the genital tract. Let us first consider low-grade, nonspecific infection being a major cause of repeat-breeding.

TABLE 1
Comparison of clinically normal repeat-breeder and first-service females

Comparison	First-service heifer [a]	Repeat-breeder	
		Heifer [b]	Cow [c]
Per cent animals with abnormalities of reproductive organs apparent at slaughter	2.7	13.5	6.0
Per cent animals showing fertilization failure, defective ova, and egg loss	21.7	35.3	39.3
Per cent animals showing embryonic loss	16.0	24.8	32.5
Per cent animals with normal embryos at 30-34 days	59.6	26.4	22.2

[a] Estimated from Kidder *et al.* (1954).
[b] Estimated from Tanabe and Almquist (1953).
[c] Estimated from Casida (1953).

LOW-GRADE NONSPECIFIC INFECTION

The infection, whether nonspecific or specific, would have to be subclinical in nature as determined by definition of the animal. Inconspicuous histopathological changes in the uterus and oviduct have been sought by various workers (7, 34, 39), but only a very low incidence of pathological changes has been noted in the absence of generalized inflammation.

The question still arises whether infections may exist and have little visible effect on the maternal tissues but yet affect the gametes or the embryo within the lumen of the genital tract. There are numerous reports (6, 9, 13, 26) that indicate an improvement in breeding performance following treatment with antibiotics, but in most of these instances either there were no controls or there was clinical evidence of an inflammation ahead of time which is ruled out in the present discussion. Where controls have been used, however, treatment of clinically normal cows by a uterine infusion of antibiotics at different stages of the estrual cycle has usually failed to increase the proportion of animals carrying normal embryos (11, 17, 24, 36, 42).

The presence of organisms in the genital tract of the living animal has been studied mostly in the anterior vagina and cervix or in the uterus by way of the cervix. The more recent of these studies of clinically normal fertile and infertile cows has shown organisms present in 25 to 40% of the animals, with virtually no indication that the presence of bacteria in the genital organs before breeding was related to subsequent fertility (11, 24).

Hawk *et al.* (15) established that the major portion of the embryonic mortality occurs soon after Day 16 of gestation. A search of the aseptically obtained uterus for microorganisms at the time that embryonic death is imminent on Day 16 should be most critical of the importance of infections as a cause of this death. Microorganisms were found, however, (14) in only 11.6% of 69 repeat-breeders at this stage, and five different kinds of organisms were represented.

Attempts also have been made without success (24) to induce lowered breeding efficiency by inoculation of heifers with organisms isolated from the genital organs of repeat-breeders. Another aspect of the problem of genital infection was considered by Menge *et al.* (33), who infected heifers repeatedly in successive estrual cycles with *Escherichia coli, Corynebacterium renale,* and *Staphylococcus aureus* toxin. These infections produced inflammatory states repeatedly, with apparent recoveries between infections. The high fertility obtained when these animals were bred failed to indicate any carry-over effects from the earlier history, which has sometimes been postulated to explain the clinically normal repeat-breeder.

POSSIBLE ENDOCRINE CAUSES

Evidence of another sort regarding the importance of infections of the genital tract in the repeat-breeder cow came unexpectedly out of apparently unrelated studies. The defense mechanisms of the uterus appear to be depressed by the action of progesterone (1, 3, 4, 30, 37). The ability of the luteal phase uterus to defend itself against infection was compared (2, 4) in heifers and in repeat-breeders. The repeat-breeders showed less evidence of an inflammatory response 24-36 hr. after inoculation and further inquiry indicated that earlier destruction of bacteria was occurring in the repeat-breeder uterus than in the virgin heifer. These observations seemed to indicate that the repeat-breeder uterus, rather than carrying a low-grade infection, may be even better able to cope with bacterial invaders than the uterus of the normal animal. A second implication of these observations is that the more effective uterine defense mechanisms in the repeat-breeder may actually indicate a functional basis for the lowered fertility, i.e., a lesser action of progesterone in these animals. This could be a lesser production of progesterone, less sensitivity of the uterus, or more rapid loss of the hormone between the source and the target organ.

The possibility that progesterone therapy might improve fertility in cows which had not settled after repeated breedings was explored by Herrick (16) and by Dawson (8). In both instances, a higher percentage of the treated animals settled than did their controls. Both sets of controls, however, showed extremely low conception rates as compared to the data earlier presented in this discussion. Wiltbank *et al.* (43), in two controlled experiments using 50 mg. progesterone daily in one trial and 200 mg. daily in the other, had better embryo survival by 11 percentage points in one trial and 13 percentage points in the other. More recently, Johnson *et al.* (20) have studied the ability of a similar treatment to improve the conception rate of first-service animals and they appear to have shown an advantage of the progesterone treatment. The conception rate of their controls, however, was extremely low.

Some attempts have now been made to get at a difference in anterior pituitary gland function which might be responsible for the repeat-breeding condition. The possibility that lactogenic hormone (presumably the luteotrophic factor) might be deficient in repeat-breeder animals was investigated by Kiddy *et al.* (22) and Cupps and Laben (7), but no demonstrable difference between them and the controls in the gland content of this hormone was

found. The content of follicle-stimulating and luteinizing hormones also has been investigated by Cupps and Laben (7), again without showing a difference between these two kinds of animals. Measurements of the progesterone content of the corpora lutea at the 14th day of the estrual cycle have also been made by Foote *et al.* (10), without any difference being demonstrated between the repeat-breeder heifers and the control heifers. Zimbelman *et al.* (44) have since shown a histological difference between the corpora lutea of pregnant and open animals at the 14th day after estrus, so that this study probably should be redone on bred animals.

The current researches having to do with a functional basis for repeat-breeding are in the areas of endocrinology and immunology. Much of the endocrine work is centered on the physiology of the corpus luteum. Further herd trials are under way testing progesterone therapy at New Jersey and South Carolina, but many of the investigations are taking on a basic character. The progesterone levels in corpora lutea representing different stages in reproduction are receiving attention. The changes during the estrual cycle have been reported by Mares and Casida (28), and a normal drop in progesterone levels of the corpora lutea during early pregnancy was reported by Zimbelman *et al.* (44). Work of Stormshak and Erb (40) suggests there may be a further rise and fall during the course of gestation. Work under way at Massachusetts and Pennsylvania is giving attention to progesterone levels in corpora lutea throughout the estrual cycle and to a comparison between repeat-breeder and normal animals. Blood levels of progesterone appear quite low at any time, but they have not been studied widely. Short (38) has indicated that these levels in the cow are remarkably constant throughout most of pregnancy, but start to decline about ten days before parturition.

Another approach in the study of the physiology of the corpus luteum is being made at Pennsylvania and at Beltsville, where the requirements for pregnancy maintenance are being determined. Minimal levels of progesterone and of estrogen for maintenance of pregnancy during the early weeks are under study in the normal and repeat-breeder animals from which the corpus luteum or the ovaries have been removed.

Still another approach to the problem is being made by experimental modification of corpus luteum function. The inhibitory effects of oxytocin and various uterine-irritating agents upon luteal maintenance have been reported by Hansel and Wagner (12). The effects of estrogen and progesterone upon corpus luteum development and function have been reported by Loy *et al.* (27) and Zimbelman *et al.* (45). Additional studies on these and other agents, in their ability to modify corpus luteum function, are under way at Cornell and Wisconsin. Work at Cornell is being related directly to embryo mortality, interference with luteal development by means of oxytocin leading to increased embryonic loss (Staples and Hansel, personal communication).

POSSIBLE IMMUNOLOGICAL CAUSES

Another possible aspect of repeat-breeding from functional causes is immunological in character. That there is a potential for interference with fertilization by antibodies was shown in rabbits by Kiddy *et al.* (23). This work was extended to cattle, with similar results, by Menge *et al.* (32). Antibodies built up against sperm experimentally, either in a different species or in the same species, were capable of preventing fertilization when brought in direct contact with the sperm in sufficient concentration.

The potential for sperm antibodies to bring about embryonic death was also shown in rabbits by Kiddy *et al.* (23). Sperm treated with a low concentration of antibodies brought about fertilization, but the resulting embryos had an increased probability of prenatal death. Menge (31) has shown that these same antibodies brought into contact with the nine-day-old embryo also increased the probability of death. Similar studies have not yet been made on embryo survival in cattle.

Whether antibodies against sperm are built up in the genital tract in nature, or whether the sperm antigens are transferred from the tract into more intimate contact with the antibody-forming system, and the antibodies in turn carried back to the genital tract, has not been studied adequately. Closely related possibilities receiving attention at Wisconsin are that embryonic loss may result (1) from certain blood antigen differences between mother and offspring or (2) from pleitropic action of genes for certain blood antigens. That the defense mechanisms of the uterus may be modified by the action of progesterone was noted earlier. The relationship of this phenomenon to possible immune responses in the uterus, and also the role that hormones may play in controlling sperm disposal in the female tract, are receiving attention.

Possible physiological relationships with repeat-breeding have come out of experimental herd studies. Lennon and Mixner (25) pointed out an association between the protein-bound iodine content of the blood plasma at 60 days postpartum and the number of services for conception. This was found significant in Holstein cows but not in Guernseys. They also reported a significant correlation between services for conception and the interval from parturition to first estrus in Guernsey cows,

but an insignificant one in Holsteins. Menge (31), however, found a significant correlation between the interval to first estrus after calving and conception on first service in an experimental herd of Holstein cows. He also found that animals that had apparently recovered from cystic ovaries within a service period still showed a lowered chance of conception on first service and an increased probability of early fetal death.

FEEDING

Lowered fertility in cattle traceable to level of feeding was reviewed by Reid (35). The percentages of heifers conceiving on first service have tended to be higher in various studies on lower planes of feeding than on higher planes, but the trends in some of the experiments tended to be biologically insignificant. Reid concluded, however, that there was a greater tendency for cows reared on a high plane of feeding to encounter breeding difficulties later in life than for cows reared on a low plane. One aspect of the feeding problem being studied today has to do with natural estrogens. Work at New Hampshire is considering the high estrogenic content of certain forages, particularly Ladino clover and the effect it may have on fertility.

HEREDITY

The genetic causes of lowered fertility were reviewed by Johansson (19) and, after summarizing the literature on heritability of number of services for conception, he concluded that selection based on this character would have a very small effect. In none of the studies for which he summarized heritabilities were data on all animals included. In all instances the cows had to conceive to be included, and this eliminates considerable variability in the beginning.

Cows of the Holstein breed have been studied more intensively than the others. Mares *et al.* (29) indicated that there was genetic variability in an experimental Holstein herd, as indicated by differences in conception rate at first service and also in early pregnancy loss among the different families. There were also effects of inbreeding, which showed up at an early stage of embryonic development when the embryo was inbred, but tended to show up at a later stage when the dam was inbred.

Studies on the differences between daughters of dams settling on first service and those of dams not settling on first service have been made on three different Wisconsin herds, two of them purebred Holstein and one a commercial herd (Table 2). Comparisons were made between the daughters of the two kinds of dams when the daughters and dams were at the same parity. The data on all first services in the herds were included. In the three herds,

TABLE 2

Comparison of breeding performance of dams and daughters

Service period	First				Second				Third				Third				All			
Dam	S		R		S		R		S		R		S		R		S		R	
Daughters	No.	% S	No.	% S	No.	% S	No.	% S	No.	% S	No.	% S	No.	% S	No.	% S	No.	% S	No.	% S
Herds:																				
WSH	67	46.3	76	34.2	37	48.6	39	33.3	17	41.2	30	40.0	15	40.0	18	33.3	136	45.6	163	35.0
P	121	61.2	83	61.4	59	57.6	42	54.8	28	57.1	27	22.2	38	50.0	13	23.1	246	58.1	165	50.3
B	175	56.0	64	53.1	94	46.8	226	39.4	81	53.1	83	55.4	82	63.4	60	51.7	432	54.9	433	46.2
All	363	55.9	223	49.8	190	50.5	307	40.7	126	52.4	140	45.7	135	57.0	91	44.0	814	54.3	761	44.7
Diff.—% S		6.1				9.8				6.7				13.0				9.6		

S = Conceived on first service.
R = Did not conceive on first service.

there was a greater chance of conception at first service and also in early pregnancy loss for the daughters of the dams that setled on first service.

Inskeep *et al.* (18) have studied differences among groups of paternal half-sisters in an artificial breeding organization for the Holstein breed and have estimated heritability at approximately 8.5%. Further studies are now under way at Wisconsin on heritability in the Guernsey breed under the conditions of artificial insemination. It is concluded that genetic variability in conception at first service is not great, but evidence for the Holstein breed, at least, points to its existence in some degree and a significant amount of variability appears to be additive in character.

DISCUSSION

A summary statement of the present status of the repeat-breeder cow problem can be brief. The problem is still with us. Our understanding of it has progressed to the place that we appreciate more and more the need for basic understanding of the reproductive physiology of the cow. The limitations of the knowledge that can be transferred from one species to another is becoming increasingly apparent. Basic knowledge is relative. It is yet to be determined whether the break-down in physiological mechanisms responsible for repeat-breeding is common to many species or whether it is in steps of the reproductive process for which there are peculiar mechanisms characteristic of the bovine.

Our knowledge of the practical handling of the repeat-breeding problem has not been greatly extended. Neither in therapy nor in management, save an appreciation of some of the harmful effects of overfeeding, is there tangible evidence of an important advance. It is the judgment of the present reviewer that the greatest opportunity for practical improvement of breeding efficiency, although admittedly it will not be rapid, lies in the genetic approach through the artificial breeding organizations. Exploration needs to be made of the possibility of gathering information on the production of live calves to first service, either as a standard procedure in the stud or on representative tester herds; any shift that then can be made in the breeding load to the sires that are leaving the more fertile daughters should result in a slow but worthwhile gain. After all, the improvement in conception rate at first service by one percentage point is economically quite important in stud operation and in the dairy production of any region.

SUMMARY

A repeat-breeder is defined as a clinically normal cow which, other factors being optimal, shows a reduced probability of conception. Both fertilization failure and embryonic loss are major causes of repeat-breeding. Much of the embryonic loss occurs between Days 16 and 25 after breeding. Nonspecific infection and undetermined after-effects, other than gross clinical pathology, have not appeared to be major causes. The uterus of the repeat-breeder during the luteal phase of the estrual cycle appears to be more resistant to bacterial invasion than that of the first-service animal, possibly indicating a deficiency of progesterone action. Therapy with progesterone, however, has not given clear-cut evidence for lessening of repeat-breeding. Poorly developed corpora lutea produced experimentally have been demonstrated to increase embryonic loss.

Experimentally produced antibodies have been shown to bring about fertilization failure and, in a somewhat lesser concentration, embryonic loss if they come in direct contact with the sperm. High planes of feeding appear to increase the incidence of breeding difficulties. Genetic causes of lowered fertility have been demonstrated, but heritability is relatively low. The degree of heritability in the Holstein breed may be high enough to give preference to certain A.I. sires on the basis of the breeding performance of their daughters.

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OUR INDUSTRY TODAY

TRENDS IN MILK FLAVOR RESEARCH [1]

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Milk flavors appear to be a common topic at our Dairy Industry Conference—every year we have one or more papers on this subject. It would be difficult to overemphasize the importance of flavor in relation to acceptability of milk and dairy products; hence, the attention it receives at our annual meeting.

Another reason that a discussion of milk flavors is always timely is that research on this subject is constantly providing new, useful information. Hence, it is necessary to reappraise this subject frequently. In this discussion I will attempt to draw your attention to some recent research trends and developments related to milk flavors, with emphasis on research at the University of California.

Role of solids-not-fat. The importance of the solids-not-fat of milk in contributing to desirable flavor was stressed last year by Stull (10) of the University of Arizona. Several research reports have recently emphasized the favorable effects of increased solids-not-fat (2, 10, 14). Some companies are now marketing products with reduced fat and increased solids-not-fat under trade names such as Two-Ten, Trimtwo, and Fortified Skimmilk.

Feed flavors. An interesting study by Dougherty and Shipe (3) is in progress at Cornell University to investigate the relative importance of the digestive tract and the respiratory tract as pathways for transmission of feed flavors to and from milk in the cow's body. The study is based on the use of cows with fistulas (trap doors) in both the trachea and the rumen. By providing independent control of transmission through the digestive and respiratory tracts, this technique permits distinguishing between the two pathways. For example, it was established that odorous gases eructated from the rumen contribute to transmission of flavors from the rumen into the milk. When selected odorous materials (ethyl and amyl acetate) were placed in the rumen through the fistula, the flavors appeared in the milk more quickly when the cows were breathing normally than when they were breathing fresh air supplied through the fistula in the trachea. Experiments such as this will provide helpful information to clarify our understanding of control of feed and respiratory flavors.

Vacuum treatments applied to milk at the time of pasteurization are receiving extensive study as an approach toward practical control of feed flavors. In a number of studies commercial equipment has been tested for its effectiveness in reducing the intensity of feed flavors. Other studies are being directed toward isolation and identification of the compounds responsible for the characteristic flavors produced from selected feeds. The objective of such studies is to provide enough information about the properties of the flavored compounds to permit predicting intensity of treatments necessary for their practical removal, or the amounts that will be removed under certain operating conditions (9). As examples of this approach, Aurand and Roberts (1) at North Carolina State College are studying feed flavor from onions and Ladino clover, and Jennings and I have recently started investigating the chemical nature of alfalfa flavor in milk.

Oxidized flavor. Many factors that influence the development of oxidized flavor in milk have been discussed in recent years at this conference. Because of the limited time, I will restrict this discussion to some recent experiments at the University of California regarding the influence of feed on susceptibility of milk to oxidized flavors.

Milk produced under the dry lot feeding system commonly used in California's market milk dairies is highly susceptible to oxidized flavor. This defect seriously limits the time that can be allowed between production and processing, and retards adoption of economies such as alternate-day milk collection and less frequent processing in the dairy plants.

Feeds are known to have a marked influence on the susceptibility of milk to oxidized flavor. For example, in a comparison made here about 3 yr. ago, milk produced on alfalfa hay was highly susceptible to oxidized flavor; whereas, that produced on a dry ration using oat hay as roughage, and also milk produced on grass pasture, was very resistant. It was thought that a detailed investigation of the differences between milks produced on the oat and the alfalfa hay rations might provide helpful information to guide producers in selecting other feeds that would yield resistant milks. In recent experiments, we found significant differences in the natural copper, carotene, and tocopherol contents of the milk, and in the oxidative stability and unsaturated fatty acid composition of the milk fat (4).

If only one constituent that might influence oxidized flavor had varied, it would have been an indication that this constituent was impor-

[1] Presented at Dairy Industry Conference, University of California, Davis, February 1, 1961.

tant in determining susceptibility of the milk to oxidized flavor. But because we found all these differences, it does not mean that each is important. Now we are conducting more feeding trials, using different rations low in certain of these constituents. We are adding the compounds we want to study in amounts equal to the difference between the oat and alfalfa hay rations, to see how much difference each constituent makes by itself.

Rancid flavor. The influence of pipe line milkers and farm tanks on the development of rancidity in milk is still being studied at a number of experiment stations. Most of the results reported recently are consistent with those obtained 5 to 10 yr. ago and, consequently, simply confirm discussions presented here previously. However, one report warrants comment, because it represents a new viewpoint.

Research conducted at Oklahoma State University emphasized that delayed or slowed cooling of milk in individual sample bottles or in farm tanks reduced the acid degree values and incidence of rancidity (7). Over 20 yr. ago, Tarassuk and Richardson (12) reported that spontaneous rancidity can be prevented by delaying the cooling of susceptible milk. The question that appears to be controversial in relation to the Oklahoma results is whether practical benefits for control of rancidity can be achieved by control of the rate of cooling and final temperature of cooling within the range that would be acceptable commercially, when considered in relation to bacterial problems. We must await further studies to clarify this point.

The influence of feed on susceptibility of milk to rancid flavor also is receiving attention. About 2 yr. ago Tarassuk and Laben (11) found that a ration consisting of alfalfa hay, silage, and grain yielded milk more susceptible to spontaneous rancidity than milk produced from a ration of pasture, silage, and grain, but there was no difference in agitation-induced rancidity. Similar results were recently reported from Connecticut—the feeding of pasture, beet pulp, and grain significantly reduced the occurrence of spontaneous rancidity as compared with that observed with alfalfa hay, beet pulp, and grain (6).

Tarassuk and Yaguchi (13) recently have obtained some very interesting results in more basic studies of the enzyme, lipase. They have used a chromatographic procedure to fractionate the proteins of milk and have found that the lipase activity is in the alpha and beta casein fractions. More information about the chemistry of lipase will undoubtedly contribute to a more complete understanding of the many factors influencing the development of rancidity in milk.

Light flavor. Light flavor should not be considered the same as oxidized flavor. It is true that under some conditions light can induce oxidized flavor by promoting oxidation of lipid constituents, but light also produces a flavor attributable to changes in milk proteins. Light absorbed by riboflavin is used to catalyze chemical changes in one or more amino acids of milk proteins (8). Hence, the light that is most troublesome is of the wave length absorbed by riboflavin—mainly in the blue region. Many factors influence the intensity of light flavor in milk, among them intensity and color of the light, time and temperature of exposure, time of storage of milk, size and material of construction of the container, homogenization, etc. The obvious control is to protect milk from light, particularly that with wave lengths below 5,000 Angstrom units. Paper and colored bottles provide good, but not complete, protection (5). Housewives should be encouraged to put milk into the refrigerator as soon as possible after delivery, or to use protected delivery boxes.

There has been concern about possible damage to milk in the brightly lighted retail cabinets in some stores. With financial assistance from American Dairy Association, we have organized a project to study this problem, but have so few results to date that none will be reported at this time. We hope that soon we will know the effect light in display cases has on milk and what can be done to eliminate damage to flavor.

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ASSOCIATION AFFAIRS

HOWARD CAMPBELL JACKSON
1892–1961

In 1920 Howard C. Jackson obtained the degree of Doctor of Philosophy at Cornell University, Ithaca, New York, where he had received his B.S. degree within a few miles of the village where he was born.

Howard Jackson's professional career began about 10 years after the founding of the American Dairy Science Association. The tremendous progress made in subsequent years in such sciences as animal physiology, nutrition, genetics, and disease control was matched by comparable strides in knowledge of the chemistry, physical chemistry, enzymology, and bacteriology of milk and its products. The 50-year history of advances in science, technology, and economics described in the JOURNAL OF DAIRY SCIENCE of June, 1956, suggests the educational skills required to master and teach this discipline during this period of progress. In these years Howard Jackson reached his full stature as scientist, teacher, counselor, and administrator.

H. C. Jackson

Beginning in 1915, Howard Jackson was an assistant and later an instructor in the Department of Dairy Industry at Cornell University. In 1920, he was made assistant professor and began work on plans for the building which houses that department at Cornell today. He organized the first course in concentrated and dried milk at Cornell and taught it until 1924, when he moved into dairy manufacturing research and extension under Dr. L. A. Rogers with the Bureau of Dairy Industry, USDA, in Washington, D. C. He was soon made manager of the dairy plant housing the experimental manufacturing laboratories of the Bureau at Grove City, Pa.

In 1927 Dean H. L. Russell appointed him chairman of the Department of Dairy Husbandry, as it was then called, at the University of Wisconsin, to succeed E. H. Farrington. This position he held with distinction under five different deans of his College for 34 years.

Professor Jackson believed in a balanced program of teaching, research, and extension. With these objectives in mind he added new members to his four-man staff. To each he offered the opportunity of independent research, with every possible aid and friendly encouragement. He promised no more than he could guarantee, yet often provided more than was expected.

His planning of improved facilities with his staff was climaxed by the construction of Babcock Hall and its occupation in 1951. It was a special delight to him that this building was named after the veteran scientist he personally knew and admired, and that the cornerstone was laid by Harry L. Russell who, after studying under Louis Pasteur, helped to make important early advances in the scientific knowledge of milk and its products.

Before World War II, Professor Jackson realized the need for courses in food technology in the University. In 1945 the Board of Regents recognized this foresight by ordering a study of the possibilities. In 1947–48, courses in food industry were first organized and taught by K. G. Weckel in the Department of Dairy Industry. Soon, the name of the Department was changed to Department of Dairy and Food Industries.

Professor Jackson's direct contributions to the research programs of his department were chiefly in arranging the financing, planning, and administration of projects. His aid was especially valuable when such projects required cooperation of other departments or organizations. His good humor smoothed the discussions of his co-workers and his stories prefaced by "Back home in New York State . . ." relaxed many tense situations. His name rarely appeared on scientific publications, but his active imagination, knowledge of trends and needs, and his friendly, helpful administration stimulated the efforts of students and staff.

The "Prof", as he was commonly known to them, was the teacher of hundreds of students during his career at Cornell and Wisconsin. He introduced these young men and women to the broad interests and responsibilities of the dairy industry in his discussions of the composition and analysis of milk and its products. He declared his personal pride in participating in an industry which served mankind so well. He was advisor to scores of students. He would whimsically ask, "Do they succeed because of what I tell them—or in spite of it?"

His qualities of popular teacher brought calls for his assistance from farm and industry. He participated in founding the American Dairy Association of Wisconsin and was its honorary director for many years. He acted on advisory committees of state government, as well as numerous University committees concerned primarily with the interests and welfare of students. His good judgment, and his practice of Christian principles endeared him to his col-

leagues and inspired genuine admiration in all who knew him.

In April, 1961, he resigned the chairmanship of his department in anticipation of his retirement in 1962. It was typical of his respect and admiration for his staff, now numbering 15, that he would not vote for a new chairman. These feelings over the years had inspired loyalty, unity, and comradeship recognized even beyond the limits of the campus.

Professor Jackson joined the American Dairy Science Association in 1923, was its Vice-President in 1931, and its President in 1933. He attended annual meetings regularly and was widely known for his friendly greeting and phenomenal memory of names and faces. He was a member of Sigma Xi, and an active member, and for many years an officer, of the Baptist Church.

In the 34 years of his work at Wisconsin only one active member of his staff had been lost by death, Hugo H. Sommer on May 8, 1953, just before the annual meeting of the American Dairy Science Association on the Wisconsin campus. It is a tragic coincidence that, just before the Association convened again in Madison, Howard C. Jackson passed away on May 8, 1961. He had just spoken in his usual informal, humorous way to a small group of friends gathered to recognize the accomplishments of one of his former students. His collapse was without warning and the end came in a few minutes while he was still unconscious.

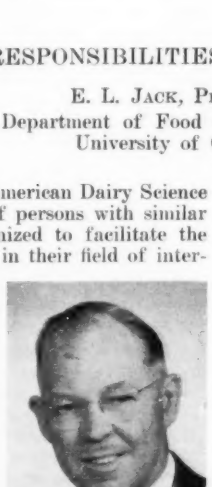
Generations of students and his contemporaries gratefully acknowledge the contributions of the life and career of this wise and friendly man.

W. V. Price
University of Wisconsin, Madison

RESPONSIBILITIES OF MEMBERSHIP

E. L. Jack, President A.D.S.A.
Department of Food Science and Technology
University of California, Davis

A society such as the American Dairy Science Association is a group of persons with similar interests who have organized to facilitate the exchange of information in their field of interest for their mutual benefit and the benefit of society at large.



E. L. Jack

The idea of benefit to the individual is uppermost in the minds of most people when they join such an organization. But if the individual is to reap the maximum benefit, the organization must be a strong one and include as many persons as possible with knowledge to be exchanged. Thus, it is evident that a member not only will benefit from association with other members, but has a responsibility, as well, to try to encourage all others of proper qualifications to join so that individual benefits may be increased.

Membership campaigns are generally organized and headed by special committees. The American Dairy Science Association has an active and aggressive membership committee. The make-up of the general committee for 1961–62 is as follows:

General Chairman—Prof. Paul M. Reaves, Virginia Polytechnic Institute, Blacksburg, Va.

Division Chairmen—

Dr. J. G. Leeder, Rutgers University, New Brunswick, N. J., Eastern Division.
Dr. Bruce Larson, University of Illinois, Urbana, Ill., Central Division.
Dr. Stanley Musgrave, Oklahoma State University, Stillwater, Southern Division.
Dr. George Stoddard, Utah State University, Logan, Western Division.
Dr. D. M. Irvine, Ontario Agricultural College, Guelph, Ontario, Canada, Central Division—Canada.
Dr. John de Man, University of Alberta, Edmonton, Alberta, Canada, Western Canada.
Dr. Roger Giroux, Department of Agriculture, Province of Quebec, St. Hyacinthe, Quebec, Canada—Eastern Canada.
Dr. W. F. Shipe, Cornell University, Ithaca, New York, Student Affiliate.

Assistant Division Chairmen—

Dr. D. J. Hankinson, University of Massachusetts, Amherst, Eastern Division.
Dr. V. H. Nielsen, Iowa State University, Ames, Central Division.
Dr. L. L. Rusoff, Louisiana State University, Baton Rouge, Southern Division.
Mr. B. E. Hubbell, Jr., University of California, Davis, Western Division.

This committee, together with state committees, is conducting a vigorous campaign to insure that every person who might benefit from membership in A.D.S.A. or who can contribute

to it will have an opportunity to join. It is not possible, however, for a committee, no matter how well organized, to cover everyone. Therefore, individual members must assist whenever they can, if the American Dairy Science Association is to remain strong and perform its proper functions.

This short note is to ask each of you individually to take personal responsibility for membership solicitation. Surely, most of you know a person or persons who should belong to A.D.S.A. Either get in touch with prospective members yourself or give their names to a member of the Committee. With increasing costs and increased Association activity it is more important than ever to maintain a full membership. It is to your personal interest to do so. Give all the help you can to the Membership Committee.

Let's make this an EVERY MEMBER GET A NEW MEMBER year.

CALL FOR PAPERS FOR THE 1962 ANNUAL MEETING OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

M. E. Senger, Chairman, Program Committee, A.D.S.A.
Department of Animal Industry, North Carolina State College, Raleigh

The 57th Annual Meeting of the American Dairy Science Association will be held June 18-21 at the University of Maryland, College Park. Members who wish to present papers must submit titles and abstracts not later than March 1. This deadline must be met to permit publication of titles with the complete program in the April, and abstracts in the June, Journal.

All members of the Association, including graduate student affiliates, are entitled to present papers. Participation by members of the industry and by senior members of the Association is especially encouraged. The Program Committee favors the general policy that an individual present only one paper and that his name appear as author on no more than two. This Committee, together with the Association membership, wishes to stimulate vigorous, sound research and realizes that these restrictions may penalize some members engaged in full-time research. Therefore, the Committee has liberalized this policy so that a member may exceed these limits, but only if he or his department rates the abstracts in order of preference for oral presentation.

Papers submitted for the annual meeting should be confined to research that has not been reported. Abstracts of papers accepted for publication by a scientific journal before the annual meeting are not eligible, as this constitutes duplication. If the total number of papers submitted by the membership is too great to include in the program, the Committee will assign some papers to be read by title only. In this event, consideration will be given to quality of research to be reported, number of abstracts per author and department, and importance of the research. Abstracts arriving late will be rejected.

Attention is called to the Dairy Manufacturing Extension Section program and members are urged to participate. This is a subsection of the Manufacturing Section.

Mimeographed copies of pertinent data are desirable for distribution when the paper is presented. At least 250 copies should be made available. This can be supplemented by slides for projection on screens, provided the author can adhere to the assigned time of 12 to 14 minutes for presentation of each paper.

PREPARING ABSTRACTS

The Program Committee encourages continued improvement in quality of papers and in oral presentation. Careful design of experiments and proper interpretation of results are necessary. Strict compliance with the instructions for preparation of abstracts will simplify the task of the Program Committee, and will improve the program. *Careful editing of abstracts before submission is essential.* Each year a number of abstracts must be returned due to incomplete details. Please follow instructions for preparing abstracts carefully.

1. All abstracts must be submitted on regular 8½- by 11-inch paper.
2. Abstracts must not exceed 200 words by actual count. Those exceeding 200 words will be returned to the author for revision.
3. An original (on bond paper) and three copies of each abstract should be typed double-spaced. The original and one copy should be mailed to the Chairman, another copy to the Vice-Chairman, and the fourth to the Secretary of the Section where the paper will be presented. The original copy will be used for publication in the Journal.
4. The style and abbreviations of the Journal of Dairy Science must be used. Please refer to abstracts in the June, 1961, Journal for guidance.
5. Only initials of authors should be used, except in unusual cases where it may be necessary to use the complete name.
6. When more than one author is listed, indi-

cate who will present the paper by an asterisk after his name.

7. The title should indicate clearly the nature of the research. It should not be repeated again in the text. The abstract should include, insofar as possible, the design and major results of the investigation. Only complete research should be reported. Brief, essential statistics will make the data more meaningful.
8. The following form with no caps for the title is correct: Utilization of carbohydrates posterior to the rumen-reticulum of the bovine. J. T. Huber and N. L. Jacobson, Iowa State University.
9. If the author lists an address for an experiment station other than the University, such as a USDA research branch or a commercial company, the complete address should be provided, as in the following example: A study of dye reduction methods as platform tests for the detection of antibiotics. Burdet Heinemann, Producers Creamery Co., Springfield, Missouri.
10. All symposium papers should be typed double-spaced and organized according to the style used in the JOURNAL OF DAIRY SCIENCE. The author should send the first copy direct to the JOURNAL Editor, E. O. Herreid, before or immediately following the Annual Meeting.

GRADUATE STUDENT PRESENTATION CONTEST

This contest will be conducted in both the Production and Manufacturing Sections. Each institution is entitled to enter one participant in each contest. They must be student affiliate members. Complete rules for the contest are being sent to department heads. Those wishing to enter the contest must submit copies of their abstract to the Section Officers as outlined above. They should also include a letter indicating their desire to enter the contest. This letter should also be signed by the major Professor and the Department Head. A carbon copy of the letter and four additional copies of the abstract should be mailed to the contest representative.

For Production Section Award: Louis Boyd, Dairy Department, University of Tennessee, Knoxville

For Manufacturing Section Award: A. V. Moore, Department of Dairy Science, Texas A&M College, College Station

Names and addresses of officers of sections to whom titles and abstracts should be sent are:

EXTENSION SECTION

Chairman: W. R. Van Sant, Department of Dairy Science, University of Arizona, Tucson.
Vice-Chairman: C. D. McGraw, Department of Dairy Science, Ohio State University, Columbus 10.
Secretary: D. E. Voelker, Department of Animal Husbandry, Iowa State University, Ames.

PRODUCTION SECTION

Chairman: J. C. Thompson, Ralston Purina Co., St. Louis, Missouri.
Vice-Chairman: L. H. Schultz, Department of Dairy Husbandry, University of Wisconsin, Madison.
Secretary: V. R. Smith, Department of Dairy Science, University of Arizona, Tucson.

MANUFACTURING SECTION

Chairman: M. L. Speck, Department of Food Science and Processing, North Carolina State College, Raleigh.
Vice-Chairman: D. M. Graham, Pet Milk Co., Research and Development Center, Greenville, Illinois.
Secretary: E. L. Thomas, Department of Dairy Industries, University of Minnesota, St. Paul.

(The Dairy Manufacturing Extension Section is a subsection of the Manufacturing Section. W. S. Arbuckle, Department of Dairy Husbandry, University of Maryland, College Park, is chairman, and A. L. Rippen, Department of Food Science, Michigan State University, East Lansing, is secretary. All abstracts for papers in this subsection should be submitted through the regular channels of the Manufacturing Section, but should be identified for presentation at the Dairy Manufacturing Extension Section meeting and a courtesy copy sent to W. S. Arbuckle.)

ABSTRACTS OF PAPERS
PRESENTED AT WESTERN DIVISION MEETING

MOSCOW, IDAHO, JULY 16–19, 1961

Payment for milk on the basis of fat and solids-not-fat. G. A. RICHARDSON, Oregon State University, Corvallis.

It was suggested the term Energy-Corrected Milk (ECM) be substituted for Fat-Corrected Milk (FCM) and that the ECM continue to be based on the energy of a 4% fat milk but also on that of a 3.3% protein milk. The basic equations, derived from data on mainly Oregon herds, are:

E/lb. = 128.9 + 52.9 f and ECM$_{(4\%)}$ = .38 M + 15.5 F.

E/lb. = 135.86 p − 108 and ECM$_{(3.5\%\,P)}$ = 40 P − .318 M

E → = Kilocalories; f and p are percentages of fat and protein; F, P, and M are pounds of fat, protein, and milk.

When equations are adjusted to conform to the fat or protein percentage of the base milk, and the price differentials determined by the percentage energy differential, or when milks are evaluated in terms of ECM, it was illustrated that the producer prices will be equitable throughout a wide range of fat and protein percentages. The milk is priced as milk without allocating prices to either fat, protein, or skimmilk. Since the protein and solids-not-fat are very highly correlated, and the mean ratio of protein to total solids is approximately constant, it was recommended that protein be included in pricing formulae. The negative sign in the protein equations discourages watering.

Some characteristics of the rennin complex. U. Y. Rege, G. A. Richardson, and J. O. Young, Oregon State University, Corvallis.

Seven fractions were isolated from commercial rennet: 1, the precipitate upon adjusting to pH 4.5; 2, precipitate after saturating supernatant with NaCl; 3, precipitate upon adjusting the latter supernatant to pH 1.5; 4, the final supernatant. Three rennet fractions, anode-wise and cathode-wise migrating, and nonmigrating, were obtained by agar electrophoresis, pH 5.8.

Hemoglobin solutions, pH 1 to 5, and a casein-sodium caseinate suspension, pH 5.2, were used as substrates for characterizing the rennet fractions on the basis of 'proteoclastic' activity, as determined spectrophotometrically (280 mμ) from 12% TCA extracts, expressed as tyrosine. All fractions were active on the hemoglobin substrate at all pH values. Greatest activity appeared in the pH range 2 to 4. The activity at pH 2 may be due to the pepsin contaminant in the extract.

With casein sols the enzyme preparations showed a rapid initial rate followed by a decreased activity. Commercial rennet exhibited the least activity. The cathode-wise and the anode-wise migrating fractions had high activities.

The milk-clotting activities of the various preparations were as follows: Rennet, 25; 1, 46; 2, 3; 3, 21; 4, 2; cathode-wise migrating, 56; anode-wise migrating, 56; nonmigrating, 98 rennin units per mg. N.

Controlled coloring of butter. G. A. Richardson and U. Y. Rege, Oregon State University, Corvallis.

A method was described and illustrated for determining the color required for producing a butter of predetermined color intensity from cream fat of unknown intensity. The method consists of isolating the clear fat by the detergent method (J.A.O.A.C., 38:931. 1955), and determining its color, in terms of carotene, by one of the following methods: (1) Simple potassium bichromate in 1.8 *M* H_2SO_4. (2) Use of a comparator and a nine-color disc corresponding to the bichromate standards (Tintometer, Ltd.). (3) Spectrophotometric, or photometric, using 10-mm. cells, at 455 mμ.

It was suggested that 1 mg. carotene be adopted as a color unit and that manufacturers of Butter Colors be exhorted to label their products in terms of this unit. This will enable the butter-maker to calculate the color required. It was suggested that 12 μg. carotene per gram of fat be considered a desirable standard for butter.

Effect on milk production of feeding different rations of alfalfa silage—alfalfa hay to dairy cattle. D. D. Miller, R. M. Porter, and S. R. Skaggs, New Mexico State University, University Park.

Four groups of three cows each were fed for 10 wk. their roughage dry matter in the following combinations: alfalfa silage 0%–alfalfa hay 100%; silage 25%–hay 75%; silage 50%–hay 50%; silage 75%–hay 25%. Digestibility of the various roughages was determined. Total digestible nutrients utilized and 4% fat-corrected milk produced were determined for two separate crop years.

Utilizing the 100% alfalfa hay ration as the standard, the 1959 crop results showed 15.4% increase and 4.2 and 6.7% decrease of 4% FCM for the 25–75, 50–50, and 75–25 rations, respectively. The TDN utilized above maintenance was 2,140 for hay alone and 1,987, 1,673, and 1,260 lb., respectively. The 1960 crop results showed 4.8, 2.3, and 0.9% increase in 4% FCM production. The TDN utilized above maintenance was 2,442, 2,427, 2,740, and 1,954 lb., respectively. Differences in individual cows and palatability of the silage accounted for the difference in TDN utilization between the two years.

Some effects caused by feeding ground, pelleted hay as compared to feeding long hay to dairy cows. D. L. Palmquist and Magnar Ronning, University of California, Davis.

Trials were conducted to observe the effects of feeding ground and pelleted hay as compared to long hay. Cows were fed long hay for 3 wk., followed in successive 3-wk. periods with hay pelleted after grinding through a hammermill with screen-hole diameters of 3/4, 3/16, or 1/16 in. Milk fat production was determined daily and the rumen was sampled via stomach tube weekly at 2 hr. post-feeding. After pH determination, the rumen samples were incubated in a Warburg respirometer to determine rates of formation of acetic, propionic, and butyric acids.

The pH of the rumen ingesta was lower and the volatile fatty acid (VFA) concen-

tration in the rumen fluid was higher in cows fed ground and pelleted hay than in those fed long hay. Rates of VFA formation were two to three times greater on the ground and pelleted hay rations than on long hay. A shift in the molar proportions among the VFA was observed only in feeding very finely ground hay, which also was associated with a depression in milk fat production.

Effects of high levels of rapeseed oil meal in dairy cow rations on milk production. J. M. ASPLUND AND L. W. MCELROY, University of Alberta, Edmonton, Alberta, Canada.

Twenty Holstein-Friesian cows were paired according to age, production, and stage of lactation. Half were fed either 10 or 20% of their total dry matter intake as rapeseed oil meal (R.S.O.M.). Pair-mates received the same levels of linseed oil meal. All cattle were fed according to production and consumed the required amounts of concentrate, even though the R.S.O.M. was obviously unpalatable. The experimental period was 12 wk. Cows receiving 10% R.S.O.M. produced slightly more milk than their controls, but the difference was not significant. Those fed 20% R.S.O.M. produced significantly less milk ($p < .05$) and declined more rapidly in production ($p < .05$) than their controls. Milk fat test was not significantly altered. There were no detectable flavor defects in the milk of cows fed R.S.O.M. that were not present in the milk of control animals.

Fat and nonfat solids as a basis for the payment for milk. L. J. MANUS, Washington State University, Pullman.

New techniques for the analysis of nonfat solids and for the protein content make it possible to switch the payment for milk from a fat and volume basis to a milk constituent basis. Emphasis must be placed on the nutritionally important protein content. Variation in the nonfat solids content of milk is mostly due to the variation of the protein content. By placing minimum standards for both fat and protein in the base milk, the pricing schedule may be made to reflect the cost factors for fat, protein, and nonfat solids minus the protein and to eliminate the total volume factor. To make this method of payment feasible for the processor, it will be necessary to change current regulations to allow the processor to standardize the final milk for protein as well as for fat. Milk in which a portion of the fat has been replaced with protein (nonfat solids) should not be sold at a discount. Formulae for developing a pricing schedule are given.

Use of milk replacers for veal production in dairy calves. A. S. HODGSON AND F. R. MURDOCK, Western Washington Experiment Station, Puyallup.

Eighteen three-day-old Holstein bull calves were used to determine the value of milk replacers for veal production. Two milk replacers were formulated which contained dried skimmilk, dried sweet cream buttermilk, and stabilized lard. In addition, each formula contained vitamins A and D, trace minerals, and oxytetracycline. One replacer formula contained 30% fat on a dry basis and the other contained 20% fat. The stabilized lard was homogenized into the mix before drying. One group of six calves received fresh whole milk; another, the milk replacer containing 30% fat; and the third group the replacer containing 20% fat. The replacers were reconstituted with warm water at 1:8 and all three groups were fed all the milk or replacer they would consume. The calves were slaughtered at approximately 42 days of age and the carcasses were graded by a federal grader. Average daily gains to slaughter were 2.58, 2.02, and 2.10 lb., respectively, and the average slaughter grades were low good, top commercial, and average commercial for each group, respectively. Calves receiving whole milk made significantly greater gains than calves receiving either milk replacer. Scouring was not a problem in any of the groups.

Preliminary observations on the use of total gas production from the artificial rumen to estimate alfalfa hay quality. R. G. HINDERS, R. E. SMITH, D. K. NELSON, AND G. M. WARD.

A rapid and simple technique to evaluate hay quality has been studied in which airtight connections were made from 50-ml. Erlenmeyer flasks to 100-ml. graduated burettes filled with water acidified to a pH of 2.4 to prevent CO_2 absorption. A 0.5-g. hay sample was incubated with 15 ml. of phosphate buffer and 25 ml. of strained rumen juice for 18 hr. at 39°C. As fermentation proceeded, the gas produced displaced the liquid in the burettes. A standard average-quality alfalfa hay was fermented in each run and gas production measurements were determined as the percentage of the gas produced by the standard alfalfa hay. Correlation coefficients between gas production and per cent crude protein, crude fiber, NFE, and cellulose digested, respectively, from 19 samples were: + 0.50, − 0.36, + 0.09, and + 0.24.

Studies of rumen parakeratosis using the chicken as a test animal. GY. VIDACS, R. G. HINDERS, AND G. M. WARD, Colorado State University, Fort Collins.

Chicks were fed a standard commercial chicken feed moistened (1:1) with the following: (I) water; (II) rumen juice from a cow which showed heavy parakeratosis on a diet of only dehydrated alfalfa pellets; (III) rumen juice from a cow fed sun-cured alfalfa hay and showing normal rumen condition; (IV) water extract of dehydrated alfalfa hay; and (V) water extract of dehydrated alfalfa pellet. Samples were taken for histological

observations from the crop, kidney, adrenal, testis, and heart; the combs were saved, also. Gain data were collected during the experiments. Parakeratosis and a thickening of the epithelium of the crop was evident not only microscopically but macroscopically on treatment (II). This indicates the crop of the chicken may be used to make qualitive observations in studies concerning rumen epithelial changes, and supports our earlier observations that rumen juice from cows with heavy parakeratosis fed to nonruminants (rats) will react upon certain stratified squamous epithelium of the nonruminant species. These observations lend strength to our hypothesis that rumen parakeratosis occurring on pelleted diets is a general reaction (physiological, metabolical, and functional) caused by some chemical factor, deficiency, or special agent.

Adaptation of the milk protein dye-binding test to field conditions. MORIO KUBOYAMA, G. A. RICHARDSON, AND J. O. YOUNG, Oregon State University, Corvallis.

Twenty-five milliliters of dye solution are placed in a 2-oz. polyethylene bottle and 0.5 ml. of milk is pipetted into the dye with a 500-lambda micropipette. The dye solution is prepared by dissolving 400 mg. of Amido Black 10-B in one liter of citrate-phosphate buffer at pH 2.3 (17.28 g. citric acid and 2.375 g. $Na_2HPO_4 \cdot 2\,H_2O$). A tube closed at one end with a fritted glass disk and flanged at the upper end is fitted through the neck of the bottle. A 0.2-ml. portion of the clear filtrate which appears in the tube is pipetted into a 10-mm. cuvette with a 200-lambda micropipette and followed by the addition of 5 ml. of distilled water. (A slight pressure by the fingertips on the flexible bottle expedites the passage of the dye solution through the fritted glass.) The per cent transmittance is obtained using a portable Coleman Model 25 photohemoglobinometer with a modified scale reading in per cent transmittance and with the usual filter replaced with a 615-mm. filter. The instrument is standardized by adjusting the reading of a dye solution containing 0.007 mg. dye per milliliter to 40% transmittance. A correlation of 0.93 was found to exist between Kjeldahl and protein values obtained by the above method in a preliminary trial using milks from 27 individual Jersey cows. A regression equation of protein = 2.26 + 0.051% T was calculated.

Use of rennet and a rennet-like substitute in Cheddar cheese manufacture. M. E. MARAGOUDAKIS, J. O. YOUNG, AND R. W. STEIN, Oregon State University, Corvallis.

Ten pairs of cheese were made with rennet and with the rennet-like substitute Metroclot. Four ounces of each enzyme preparation was used per 1,000 lb. of milk with all other factors being kept as much alike as possible. The soluble tyrosine and tryptophone, as estimated from the optical density of the cheese extract in a sodium citrate-HCl solution, was higher in the rennet cheese at 30, 60, 90, and 150 days. The same was true of the amino nitrogen content as measured by the formol titration. All differences were significant at the 1% level. The mean values of organoleptic tests performed by five expert judges indicated that the rennet cheese had a more desirable body and texture, greater flavor intensity, a more desirable flavor, and a better over-all score after 30, 60, and 90 days. At 150 days, however, the cheese made with the substitute had a more desirable flavor. This was due to a marked improvement in the substitute cheese and a decline in quality of the rennet cheese. Bitterness was more prevalent in the rennet cheese at all age intervals tested. A high correlation was found to exist between soluble tyrosine or tryptophane content and flavor intensity and between tyrosine or tryptophane content and bitterness. Amino nitrogen content and pH values were highly correlated with flavor intensity only.

Relationships of date of cutting, stage of maturity, and digestibility of orchardgrass. F. R. MURDOCK, A. S. HODGSON, AND J. R. HARRIS, Western Washington Experiment Station, Puyallup.

Orchardgrass forage was harvested daily and fed to four yearling dairy heifers beginning April 22 and continuing through June 6. Dry matter digestibility was determined at four-day intervals by means of fecal chromogen analyses. Dry matter digestibility remained fairly constant at approximately 75% until May 5 or early boot stage. Digestibility declined very rapidly from 72.2% on May 9 to 60.8% on May 21. The average decline in digestibility during this 12-day period, during which the forage advanced from the boot to nearly full head stage, was approximately 1% per day. Dry matter digestibility continued to decline through successive stages until June 6, but at a slower rate than at the early head stage. The data suggest a curvilinear relationship between dates of cutting and dry matter digestibility of orchardgrass forage.

Comparison of different pasture grazing proccedures and green chop upon milk and fat production. D. L. THACKER, R. H. ROSS, AND D. L. FOURT, Caldwell Branch Experiment Station, Caldwell, Idaho, and University of Idaho, Moscow.

Twelve acres of irrigated Ladino clover orchardgrass pasture were divided into plots to be grazed or chopped. In one-half of the four replicates, Latar orchardgrass was used to compare with common orchardgrass. In addition, three acres were seeded to an all-Ladino clover pasture. The management practices studied were: (1) Rotational grazing, cows had the whole plot for seven days, (2)

strip grazing, cows had fresh grass daily, (3) rationed grazing, cows had fresh grass three times a day, (4) green chop, (5) all–Ladino clover rationed grazed. The cows were randomly assigned to one of the above treatments for the entire grazing season. The 2-yr. average production of 4% F.C.M. per acre was: (1) 7,014 lb. (2) 6,763 lb. (3) 6,775 lb. (4) 5,995 lb. (5) 7,353 lb. The 2-yr. average cow-days grazing per acre was: (1) 218, (2) 218, (3) 222, (4) 169, (5) 213, in a grazing season of 154 days. The cows under all management systems produced 6,636 lb. of F.C.M. in 211 cow-grazing days per acre while on common orchardgrass, and 6,577 lb. of F.C.M. in 203 cow-grazing days per acre while on Latar orchardgrass. The botanical composition of the forage available for grazing in the months of July and August has changed in the 2 yr. from 70% Ladino clover, first year, to about 10%, second year, in the three grazing management studies. In the plots green-chopped, it changed from 85 to 32%. All-Ladino clover has changed from 88 to 72%. The amount of grass has increased slightly more than the decrease in Ladino clover. There are fewer weeds in all plots except the all-Ladino clover plots. Weeds have increased from 2 to 17% in these plots. Bloat has not been a problem during the 2-yr. study.

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BOOK REVIEW

FOETAL AND NEONATAL PHYSIOLOGY. British Medical Bulletin 17(2): 79-176. 1961. The British Council, 65 Davies Street, London W.1. $3.25.

This issue of the Bulletin is made up of a group of articles, each written by an authority in his field, covering the various phases of foetal and neonatal physiology. The articles are comprehensive yet brief and up-to-date. The areas are indicated by the titles of the articles: histology of the placenta, foetal endocrinology, protein transfer across foetal membranes, immunological tolerance and paralysis, transport mechanisms in the foetus, foetal kidney, enzymic activity during early development, carbohydrate metabolism in placenta and foetus, inferior aortico-pulmonary glomus, perinatal mineral metabolism, glycogen reserves and their changes at birth, ability of young mammals to withstand total oxygen lack, changes in the circulation at birth, blood pressure in the new-born baby, respiration in the new-born baby, the new-born animal and environmental temperature, neonatal paediatries, and animal physiology.

R. L. HAYS
University of Illinois, Urbana

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